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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/00, 38/46, 48/00, C07K 2/00, 4/00, 14/00, 16/00, C12N 15/57

A1

(11) International Publication Number: **WO 96/20721**

(43) International Publication Date: 11 July 1996 (11.07.96)

(21) International Application Number: PCT/US96/00177

(22) International Filing Date: 4 January 1996 (04.01.96)

(30) Priority Data:
08/368,704 4 January 1995 (04.01.95) US

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(81) Designated States: AU, CA, FI, JP, MX, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROGRAMMED CELL DEATH GENES AND PROTEINS

(57) Abstract

This invention relates to genes involved in regulating programmed cell death, the proteins encoded by such genes and methods for controlling programmed cell death by regulating the activity of the cell death gene products.

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Programmed Cell Death Genes and Proteins

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Cross-Reference to Related Applications

This application is a continuation-in-part application of U.S. Application No. 08/258,287, filed June 10, 1994, which is a continuation-in-part application of U.S. Application No. 08/080,850, filed June 24, 1993, now abandoned.

Field of the Invention

The invention is in the field of molecular biology as related to the control of programmed cell death.

Description of the Background Art

Programmed Cell Death

Cell death occurs as a normal aspect of animal development as well as in tissue homeostasis and aging (Glucksmann, A., *Biol. Rev. Cambridge Philos. Soc. 26*:59-86 (1950); Ellis *et al.*, *Dev. 112*:591-603 (1991); Vaux *et al.*, *Cell 76*:777-779 (1994)). Naturally occurring cell death acts to regulate cell number, to facilitate morphogenesis, to remove harmful or otherwise

abnormal cells and to eliminate cells that have already performed their function. Such regulated cell death is achieved through an endogenous cellular mechanism of suicide, termed programmed cell death or apoptosis (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34). Programmed cell death or apoptosis occurs when a cell activates this internally encoded suicide program as a result of either internal or external signals. The morphological characteristics of apoptosis include plasma membrane blebbing, condensation of nucleoplasm and cytoplasm and degradation of chromosomal DNA at inter-nucleosomal intervals. (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34). In many cases, gene expression appears to be required for programmed cell death, since death can be prevented by inhibitors of RNA or protein synthesis (Cohen *et al., J. Immunol. 32*:38-42 (1984); Stanisic *et al., Invest. Urol. 16*:19-22 (1978); Martin *et al., J. Cell Biol. 106*:829-844 (1988)).

The genetic control of programmed cell death has been well-elucidated by the work on programmed cell death in the nematode *C. elegans*. Programmed cell death is characteristic and widespread during *C. elegans* development. Of the 1090 somatic cells formed during the development of the hermaphrodite, 131 undergo programmed cell death. When observed with Nomarski microscopy, the morphological changes of these dying cells follow a characteristic sequence. (Sulston *et al., Dev. Biol. 82*:110-156 (1977); Sulston *et al., Dev. Biol. 100*:64-119 (1983)).

Fourteen genes have been identified that function in different steps of the genetic pathway of programmed cell death in *C. elegans* (Hedgecock *et al., Science 220*:1277-1280 (1983); Ellis *et al., Cell 44*:817-829 (1986); Ellis *et al., Dev. 112*:591-603 (1991); Ellis *et al., Genetics 112*:591-603 (1991b); Hengartner *et al., Nature 356*:494-499 (1992); Ellis *et al., Dev. 112*:591-603 (1991)). Two of these genes, *ced-3* and *ced-4*, play essential roles in either the initiation or execution of the cell death program. Recessive

mutations in these genes prevent almost all of the cell deaths that normally occur during *C. elegans* development.

The *ced-4* gene encodes a novel protein that is expressed primarily during embryogenesis, the period during which most programmed cell deaths occur (Yuan *et al., Dev. 116*:309-320 (1992)). The 549 amino acid sequence of ced-4, deduced from cDNA and genomic clones, contain two regions that are similar to the calcium-binding domain known as the EF-hand (Kretsinger, 1987); however, it is still not clear at present whether calcium plays a role in regulating ced-4 or programmed cell death in *C. elegans*.

A gain-of-function mutation in *ced-9* prevents normal programmed cell death, while mutations that inactivate *ced-9* are lethal. This suggests that *ced-9* acts by suppressing programmed cell death genes in cells that normally do not undergo programmed cell death (Hengartner, M., *et al., Nature 356*:494-499 (1992)). The *ced-9* gene encodes a protein product that shares sequence similarity with the mammalian proto-oncogene and cell death suppressor *bcl-2* (Hengartner, M., *et al., Cell 76*:665-676 (1994)). The lethality of *ced-9* loss-of-function mutations can be suppressed by mutations in *ced-3* and *ced-4*, indicating that *ced-9* acts by suppressing the activity of *ced-3* and *ced-4*.

Genetic mosaic analyses indicate that *ced-3* and *ced-4* likely act in a cell-autonomous fashion within dying cells, suggesting that they might be cytotoxic proteins and/or control certain cytotoxic proteins in the process of programmed cell death (Yuan, J., *et al., Dev. Bio. 138*:33-41 (1990)).

nedd2

Cell death also occurs in mammals. *nedd2* is a mouse gene which is preferentially expressed during early embryonic brain development (Kumar *et al., Biochem. Biophys. Res. Commun. 185*:1155-1161 (1992)). Since many neurons die during early embryonic brain development, it is possible that *nedd*-2 is a cell death gene. Nedd-2 mRNA is down-regulated in the adult

brain (Kumar *et al.*, *Biochem. & Biophys. Res. Comm. 185*:1155-1161 (1992); Yuan, J., *et al.*, *Cell 75*:641-752 (1993)).

bcl-2

bcl-2 is an oncogene known to inhibit programmed cell death and to be overexpressed in many follicular and B cell lymphomas. Overexpression of *bcl-2* as a result of chromosomal translocation occurs in 85% of follicular and 20% of diffuse B cell lymphomas, Fukuhara *et al.*, *Cancer Res. 39*:3119 (1979); Levine *et al.*, *Blood 66*:1414 (1985); Yunis *et al.*, *N. Engl. J. Med. 316*:79-84 (1987).

Overexpression of *bcl-2* protects or delays the onset of apoptotic cell death in a variety of vertebrate cell types as well as in *C. elegans* (Vaux *et al.*, *Science 258*:1955-1957 (1992); Nunez *et al.*, *J. Immun. 144*:3602-3610 (1990); Vaux *et al.*, *Science 258*:1955-1957 (1992); Sentman *et al.*, *Cell 67*:879-888 (1992); Strasser *et al.*, *Cell 67*:889-899 (1991)). Expression of *bcl-2* in the nematode *C. elegans* has been shown to partially prevent programmed cell death. Thus, *bcl-2* is functionally similar to the *C. elegans ced-9* gene (Vaux *et al.*, *Science 258*:1955-1957 (1992); Hengartner *et al.*, *nature 356*:494-499 (1992)).

Interleukin-1β Converting Enzyme

Interleukin-1β converting enzyme (ICE) is a substrate-specific cysteine protease that cleaves the inactive 31 KD prointerleukin-1β at Asp^{116}-Ala^{117}, releasing a carboxy-terminal 153 amino-acid peptide to produce the mature 17.5 kD interleukin-1β (IL-1β) (Kostura *et al.*, *Proc. Natl. Acad. Sci., USA 86*:5227-5231 (1989); Black *et al.*, *FEBS Lett. 247*:386-390 (1989); Cerretti *et al.*, *Science 256*:97-100 (1992); Thornberry *et al.*, *Nature 356*:768-774 (1992)). IL-1β is a cytokine involved in mediating a wide range of biological responses including inflammation, septic shock, wound healing, hematopoiesis

and growth of certain leukemias (Dinarello, C.A., *Blood 77*:1627-1652 (1991); diGiovine *et al., Today 11*:13 (1990)). A specific inhibitor of ICE, the *crmA* gene product of cowpox virus, prevents the proteolytic activation of IL-1β (Ray *et al., Cell 69*:597-604 (1992)) and inhibits host inflammatory response (Ray *et al., Cell 69*:597-604 (1992)). Cowpox virus carrying a deleted *crmA* gene is unable to suppress the inflammatory response of chick embryos, resulting in a reduction in the number of virus-infected cells and less damage to the host (Palumbo *et al., Virology 171*:262-273 (1989)). This observation indicates the importance of *ICE* in bringing about the inflammatory response.

Tumor Necrosis Factor

Tumor necrosis factor-α (TNF-α) is a pleiotropic tumoricidal cytokine (Tracey, K.J. *et al., Ann. Rev. Cell. Biol. 9*:317-343 (1993)). One of the striking functions of TNF-α is to induce apoptosis of transformed cells. In the case of non-transformed cells, TNFα can also induce apoptosis in the presence of metabolic inhibitors (Tracey, K.J., *et al., Ann. Rev. Cell. Biol. 9*:317-343 (1993). Apoptosis induced by TNF-α is also suppressed by *bcl-2*.

One of the most extensively studied functions of TNF-α is its cytotoxicity on a wide variety of tumor cell lines *in vitro* (Laster, S. M. *et al., J. Immunol. 141*:2629-2634 (1988)). However, the mechanism of cell death induced by TNF has been largely unknown. HeLa cells express predominantly p55 TNF receptor which is thought to be responsible for cell death signaling (Englemann, H. *et al., J. Biol. Chem. 265*:14497-14504 (1990); Thoma, B. *et al., J. Exp. Med. 172*:1019-1023 (1990)).

HeLa cells are readily killed by TNF-α in the presence of the metabolic inhibitor cycloheximide (CHX). The cell death induced by TNF-α/CHX shows DNA fragmentation and cytolysis, which are typical features of apoptosis (White, E. *et al., Mol. Cell. Biol. 12*:2570-2580 (1992)). Expression of adenovirus E1B 19K protein, which is functionally similar to

bcl-2, inhibits apoptosis induced by TNF in HeLa cells (White, E. *et al.*, *Mol. Cell. Biol. 12*:2570-2580 (1992)).

Summary of the Invention

In the present invention, the *ced-3* gene has been cloned and sequenced and the amino acid sequence of the protein encoded by this gene is disclosed. Structural analysis of the *ced-3* gene revealed that it is similar to the enzyme interleukin-1β converting enzyme ("ICE") and that overexpression of the murine interleukin-1β converting enzyme ("mICE") causes programmed cell death in vertebrate cells. Based upon these results, a novel method for controlling programmed cell death in vertebrates by regulating the activity of *ICE* is claimed.

The amino acid sequence of ced-3 was also found to be similar to another murine protein, nedd-2, which is detected during early embryonic brain development, a period when many cells die. The results suggest that *ced-3*, *ICE* and *nedd-2* are members of a gene family which function to cause programmed cell death.

A new cell death gene, *mIch-2* (murine *ICE-ced-3* homolog), has been discovered which appears to be in the same family as *ced-3*, *ICE*, and *nedd-2*. *mIch-2* is distinguished from other previously identified cell death genes in that it is preferentially expressed in the thymus and placenta of vertebrates. Thus, the invention is also directed to a newly discovered gene, *mIch-2*, which is preferentially expressed in thymus and placenta and which encodes a protein causing programmed cell death. Thus, the present invention is directed to both the *mIch-2* gene sequence and the protein encoded by *mIch-2*. Also encompassed are vectors expressing *mIch-2* and host cells transformed with such vectors. The invention also encompasses methods of regulating cell death by expressing *mIch-2*.

A comparison of the nucleotide sequences of *ced-3*, *mICE*, human *ICE* ("*hICE*"), *nedd-2* and *mIch-2* indicates that they are members of a gene family

that promotes programmed cell death. The identification of this family facilitated the isolation of the newly discovered cell death gene *Ice-ced 3* homolog-1 (*Ich-1*).

Ich-1 is homologous to the other cell death genes described above and particularly to *nedd2*. Based upon its structure and the presence of DNA encoding the QACRG sequence characteristic of the active center of cell death genes, *Ich-1* was identified as a new member of the *ced-3/ICE* family. Thus, the present invention is directed to both the *Ich-1* gene sequence and the protein encoded by *Ich-1*. Also encompassed are vectors expressing *Ich-1* and host cells transformed with such vectors. Alternative splicing results in two distinct *Ich-1* mRNA species. Thus, the invention also encompasses these species, proteins produced from them, vectors containing and expressing the genes, and the uses described herein.

The inventors have also identified a further member of the *ICE/ced-3* family, *Ich-3*. *Ich-3* has at least two alternative splicing products. A full length cDNA of one of them from a mouse thymus cDNA library has been identified. It encodes a protein of 418 amino acids that is 38% identical with mICE, 42% identical with mIch-2, 25% with Ich-1, and 24% identical with *C. elegans* ced-3.

The invention is thus directed to genomic or cDNA nucleic acids having genetic sequences which encode ced-3, mIch-2, Ich-1, and Ich-3. The invention also provides for vectors and expression vectors containing such genetic sequences, the host cells transformed with such vectors and expression vectors, the recombinant nucleic acid or proteins made in such host/vectors systems and the functional derivatives of these recombinant nucleic acids or proteins. The use of the isolated genes or proteins for the purpose of regulating, and especially promoting cell death is also part of the invention.

The invention is also directed to methods for controlling the programmed death of vertebrate cells by regulating the activity of interleukin-1β converting enzyme. Such regulation may take the form of inhibiting the enzymatic activity, e.g. through the use of specific antiproteases such as

crmA, in order to prevent cell death. In this way, it may be possible to develop cell lines which remain viable in culture for an extended period of time or indefinitely. Certain cells can only be maintained in culture if they are grown in the presence of growth factors. By blocking cell death, it may be possible to make such cells growth factor independent. Alternatively, ICE activity may be increased in order to promote cell death. Such increased activity may be used in cancer cells to antagonize the effect of oncogenes such as *bcl-2*.

The present invention is also based on the discovery that TNF-α activates endogenous ICE activity in HeLa cells and that TNF-α-induced apoptosis is suppressed by crmA, which can specifically inhibit ICE-mediated cell death. Thus, certain embodiments of the invention are based on the activation of the *ICE/ced-3*-mediated cell death pathway by TNF-α.

Brief Description of the Figures

Figure 1 and 1A: Genetic and Physical Maps of the ced-3 Region on Chromosome IV

Figure 1 shows the genetic map of *C. elegans* in the region near *ced-3* with the cosmid clones representing this region depicted below the map. *nP33, nP34, nP35, nP36,* and *nP37* are restriction fragment length polymorphisms (RFLP) between Bristol and Bergerac wild type *C. elegans* strains. C43C9, W07H6 and C48D1 are three cosmid clones tested for rescue of the *ced* phenotype of *ced-3(n717)* animals. The ability of each cosmid clone to rescue *ced-3* mutants and the fraction of independently obtained transgenic lines that were rescued are indicated on the right of the figure (+, rescue; -, no rescue; see text for data). The results indicate that *ced-3* is contained in the cosmid C48D1.

Figure 1A is a restriction map of C48D1 subclones. C48D1 was digested with *Bam*HI and self-ligated to generate subclone C48D1-28.

C48D1-43, pJ40 and pJ107 were generated by partial digesting C48D1-28 with *Bgl*II. pJ7.5 and pJ7.4 were generated by *Exo*III deletion of pJ107. These subclones were assayed for rescue of the *ced* phenotype of *ced-3(n717)* animals (+, rescue; -, no rescue, -/+, weak rescue). The numbers in parentheses indicate the fraction of independently obtained transgenic lines that were rescued. The smallest fragment that fully rescued the *ced-3* mutant phenotype was the 7.5 kb pJ7.5 subclone.

Figure 2, 2A(i)-2A(v), 2B and 2C:
Genomic Organization, Nucleotide Sequence, and Deduced Amino Acid Sequence of ced-3

Figure 2 shows the genomic sequence of the *ced-3* region, as obtained from plasmid pJ107. The deduced amino acid sequence of ced-3 is based on the DNA sequence of *ced-3* cDNA pJ87 and upon other experiments described in the text and in Experimental Procedures. The 5' end of pJ87 contains 25 bp of poly-A/T sequence (not shown), which is probably a cloning artifact since it is not present in the genomic sequence. The likely start site of translation is marked with an arrowhead. The SL1 splice acceptor site of the *ced-3* transcript is boxed. The positions of 12 *ced-3* mutations are indicated. Repetitive elements are indicated as arrows above the relevant sequences. Numbers on the left indicate nucleotide positions, beginning with the start of pJ107. Numbers below the amino acid sequence indicate codon positions. Five types of imperfect repeats were found: repeat 1, also found in *fem-1* (Spence *et al.*, *Cell 60*:981-990 (1990)) and *hlh-1* (Krause *et al.*, *Cell 63*:907-919 (1990)); repeat 2, novel; repeat 3, also found in *lin-12* and *fem-1*; repeat 4, also found in *lin-12*; and repeat 5, novel. Numbers on the sides of the figure indicate nucleotide positions, beginning with the start of pJ107. Numbers under the amino acid sequence indicate codon positions.

Figure 2A(i) - Figure 2A(iv) contain comparisons of the repetitive elements in *ced-3* with the repetitive elements in the genes *ced-3*, *fem-1*, *hlh-1*,

lin-12, *glp-1*, and the cosmids B0303 and ZK643 (see text for references). In the case of inverted repeats, each arm of a repeat ("for" or "rev" for "forward" or "reverse", respectively) was compared to both its partner and to individual arms of the other repeats. 2A(i): Repeat 1; 2A(ii): Repeat 2; 2A(iii): Repeat 3; 2A(iv): Repeat 4; and 2A(v): Repeat 5. The different *ced-3* sequences which appear in the comparisons are different repeats of the same repetitive element. The numbers "1a", "1b" etc. are different repeats of the same class of repetitive element.

Figure 2B shows the locations of the introns (lines) and exons (open boxes) of the *ced-3* gene as well as the positions of 12 *ced-3* mutations analyzed. The serine-rich region, the trans-spliced leader (SL1), the possible start of translation (ATG) and polyadenlyation (AAA) site are also indicated.

Figure 2C shows the cDNA sequence and deduced amino acid sequence of *ced-3* as obtained from plasmid pJ87.

Figure 3 and 3A: Comparison of the Structure of the ced-3 Protein and hICE Protein

Figure 3 shows a comparison of structural features of *ced-3* with those of the *hICE* gene. The predicted proteins corresponding to the hICE proenzyme and *ced-3* are represented. The active site in hICE and the predicted active site in ced-3 are indicated by the black rectangles. The four known cleavage sites in hICE flanking the processed hICE subunits (p24, which was detected in low quantities when hICE was purified (Thornberry *et al.* (1992), p20, and p10) and two conserved presumptive cleavage sites in ced-3 are indicated with solid lines and linked with dotted lines. Five other potential cleavage sites in ced-3 are indicated with dashed lines. The positions of the aspartate (D) residues at potential cleavage sites are indicated below each diagram.

Figure 3A contains a comparison of the amino acid sequences of ced-3 from *C. elegans*, *C. briggsae* and *C. vulgaris* with hICE, mICE, and mouse

nedd-2. Amino acids are numbered to the right of each protein. Dashes indicate gaps in the sequence made to allow optimal alignment. Residues that are conserved among more than half of the proteins are boxed. Missense *ced-3* mutations are indicated above the comparison blocks showing the residue in the mutant ced-3 and the allele name. Asterisks indicate potential aspartate self-cleavage sites in ced-3. Circles indicate known aspartate self-cleavage sites in hICE. Residues indicated in boldface correspond to the highly conserved pentapeptide containing the active cysteine.

Figure 4:
Construction of Expression Cassettes of mICE-lacZ and ced-3-lacZ Fusion Genes

Figure 4 shows several expression cassettes used in studying the cellular effects of *ICE* and *ced-3* expression. The cassettes are as follows: pβactM10Z contains intact *mICE* fused to the *E. coli lacZ* gene (*mICE-lacZ*). pβactM11Z contains the P20 and P10 subunits of *mICE* fused to the *E. coli lacZ* gene (P20/P10-*lacZ*). pβactM19Z contains the P20 subunit of *mICE* fused to the *E. coli lacZ* gene (P20-*lacZ*). pβactM12Z contains the P10 subunit of *mICE* fused to the *E. coli lacZ* gene (P10-*lacZ*). pβactced38Z contains the *C. elegans ced-3* gene fused to the *lacZ* gene (*ced-3-lacZ*). pJ485 and pβactced37Z contain a Gly to Ser mutation at the active domain pentapeptide "QACRG" in mICE and ced-3 respectively. pβactM17Z contains a Cys to Gly mutation at the active domain pentapeptide "QACRG" in mICE. pactβgal' is a control plasmid (Maekawa *et al.*, *Oncogene* 6:627-632 (1991)). All plasmids use the β-actin promoter.

Figure 5:
Genetic Pathways of Programmed Cell Death in the Nematode C. elegans and in Vertebrates

In vertebrates, bcl-2 blocks the activity of ICE thereby preventing programmed cell death. Enzymatically active ICE causes vertebrate cell death. In *C. elegans*, ced-9 blocks the action of ced-3/ced-4. Active ced-3 together with active ced-4 causes cell death.

Figure 6:
mIch-2 cDNA Sequence and Deduced Amino Acid Sequence

Figure 6 shows the nucleotide sequence of the *mIch-2* cDNA sequence and the amino acid sequence deduced therefrom.

Figure 7 and 7A:
mIch-2 Amino Acid Sequence

Figures 7 and 7A contain a comparison of the amino acid sequences of mICE, hICE, mIch-2 and ced-3.

Figure 8:
Potential QACRG Coding Region in the Mouse nedd2 cDNA

The reading frame proposed by Kumar *et al.* (*Biochem. & Biophys. Res. Comm. 185:*1155-1161 (1992)) is b. In reading frame a, a potential QACRG coding region is underlined.

Figure 9 and 9A:
Comparison of the Amino Acid Sequences of ced-3, hICE and Ich-1

Figure 9 contains a comparison of the amino acid sequences of ced-3 and Ich-1. There is a 52% similarity between the sequences and a 28% identity.

Figure 9A contains a comparison of the amino acid sequences of hICE and Ich-1. There is a 52% similarity between the sequences and a 27% identity.

Figure 10A:
The cDNA Sequence of Ich-1_L and the Deduced Amino Acid Sequence of Ich-1_L Protein Product

The putative active domain is underlined.

Figure 10B:
The cDNA Sequence of Ich-1_S and the Deduced Amino Acid Sequence of Ich-1_S Protein Product

The intron sequence is underlined.

Figure 11A:
The Alternative Splicing of Ich-1 mRNA

The exons are shown in bars. The intron is shown in a line. Nucleotides at the exon-intron borders are indicated.

Figure 11B:
The Schematic Diagram of the Two Alternatively Spliced Ich-1 Transcripts and Ich-1_L and Ich-1_S Proteins

The peptide marked with X may not be translated *in vivo*. The open reading frames and proteins are shown in bars. Untranslated regions and

introns are shown in lines. The positions of Ich-1_S and Ich-1_L stop codons are indicated on the transcript diagram as "stop". Amino acid sequences in Ich-1_S that are different from Ich-1_L are shaded.

Figure 12A:
A Comparison of the Ich-1 Protein Sequence with the Mouse nedd-2 Protein, the hICE Protein, the mICE Protein, and C. elegans ced-3 Protein

Amino acids are numbered to the right of each sequence. Any residues in nedd-2, hICE, mICE, and ced-3 that are identical with Ich-1 are highlighted. The two point mutations made by site-directed mutagenesis are marked on the top of the sequence.

Figure 12B:
A Schematic Comparison of Structural Features of Ich-1_L and hICE

The active site of hICE and predicted active site of Ich-1_L are indicated. The four known cleavage sites of hICE and potential cleavage sites of Ich-1_L are marked.

Figure 13:
Stable Expression of Ich-1_S Prevents Cell Death of Rat-1 Cells Induced by Serum Removal

Stable transfectants of Rat-1 cells expressing *bcl-2*, *crmA* or *Ich-1_S* were prepared as described in Experimental Procedures. Independent *Ich-1_S*-positive and *Ich-1_S*-negative clones were used. At time 0, exponentially growing cells were washed with serum-free DMEM and dead cells were counted over time by trypan blue staining.

Figure 14:
The cDNA Sequence and Putative Ich-3 Protein Sequence

The putative first Met is marked with a dot.

Figure 15:
Comparison of Amino Acid Sequences of Ich-3 with hICE, mIch-2, Ich-1 and ced-3

Figure 16:
Suppression of TNF-Induced Cytotoxicity by Overexpression of CrmA

The results are from three separate experiments with each condition done in duplicate.

Definitions

In the description that follows, a number of terms used in recombinant DNA (rDNA) technology or in the research area of programmed cell death are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Gene. A DNA sequence containing a template for a RNA polymerase. The RNA transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA). It is understood, however, that a gene also encompasses non-transcribed regulatory sequences including, but not limited to, such sequences as enhancers, promoters, and poly-A addition sequences.

A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening

sequences (introns) have been removed. Of course, cDNA may also include any complementary part of any gene sequence. The complement could be synthesized, for example, and may not exclude DNA sequences not found in the naturally occurring mRNA.

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance. The term "cloning vehicle" is sometimes used for "cloning vector."

Expression vector. A vector similar to a cloning vector but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

Programmed cell death. The process in which cell death is genetically programmed. Programmed cell death allows organisms to eliminate cells that have served a developmental purpose but which are no longer beneficial.

Functional Derivative. A "functional derivative" of *mIch-2*, *Ich-1* (*Ich-1*$_L$ and *Ich-1*$_S$), or *Ich-3* is a protein, or DNA encoding a protein, which possesses a biological activity that is substantially similar to the biological activity of the non-recombinant. A functional derivative of may or may not contain post-translational modifications such as covalently linked carbohydrate,

depending on the necessity of such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. The derivative retains at least one of the naturally-occurring functions of the parent gene or protein. The function can be any of the regulatory gene functions or any of the function(s) of the finally processed protein. The degree of activity of the function need not be quantitatively identical as long as the qualitative function is substantially similar.

Fragment. A "fragment" is meant to refer to any subgenetic sequence of the molecule, such as the peptide core, or a variant of the peptide core.

Detailed Description of the Preferred Embodiments

Description

The present invention relates, *inter alia*, to isolated DNA encoding ced-3 of *C. elegans*, mIch-2, Ich-1, and Ich-3. The invention also encompasses nucleic acids having the cDNA sequence of *ced-3*, *mIch-2*, *Ich-1*, and *Ich-3*. The invention also encompasses related sequences in other species that can be isolated without undue experimentation. It will be appreciated that trivial variations in the claimed sequences and fragments derived from the full-length genomic and cDNA genes are encompassed by the invention as well. The invention also encompasses protein sequences encoded by *ced-3*, *mIch-2*, *Ich-1*, and *Ich-3*. It should also be understood that *Ich-1* encompasses both *Ich-1$_S$ and Ich-1$_L$*.

ced-3

The genomic sequence of the claimed gene encoding ced-3 is shown in Figure 2. The gene is 7,656 base pairs in length and contains seven introns ranging in size from 54 base pairs to 1,195 base pairs. The four largest

introns as well as sequences 5′ to the START codon contain repetitive elements, some of which have been previously characterized in the non-coding regions of other *C. elegans* genes such as *fem-1* (Spence *et al.*, *Cell 60*:981-990 (1990)) and *hlh-1* (Krause *et al.*, *Cell 63*:907-919 (1990)). A comparison of the repetitive elements in *ced-3* with previously characterized repetitive elements is shown in figures 2A(i) - 2A(v). The START codon of ced-3 is the methionine at position 2232 of the genomic sequence shown in Figure 2.

The cDNA sequence of *ced-3* shown in Figure 2C. The cDNA is 2,482 base pairs in length with an open reading frame encoding 503 amino acids and 953 base pairs of 3′ untranslated sequence. The last 380 base pairs of the 3′ sequence are not essential for the expression of *ced-3*.

In addition to encompassing the genomic and cDNA sequences of *ced-3* from *C. elegans*, the present invention also encompasses related sequences in other nematode species which can be isolated without undue experimentation. For example, the inventors have shown that *ced-3* genes from *C. briggsae* and *C. vulgaris* may be isolated using the *ced-3* cDNA from *C. elegans* as a probe (see Example 1).

The invention also encompasses protein products from the *ced-3* gene, gene variants, derivatives, and related sequences. As deduced from the DNA sequence, ced-3 is 503 amino acids in length and contains a serine-rich middle region of about 100 amino acids. The amino acid sequence comprising the claimed ced-3 is shown in Figure 2 and Figure 2C. A comparison of ced-3 of *C. elegans* with the inferred ced-3 sequences from the related nematode species *C. briggsae* and *C. vulgaris* indicates that the non-serine-rich region is highly conserved and that the serine-rich region is more variable. The non-serine-rich portion of ced-3 is also homologous with hICE. The C-terminal portions of both ced-3 and hICE are similar to the mouse nedd-2. The results suggest that ced-3 acts as a cysteine protease in controlling the onset of programmed cell death in *C. elegans* and that members of the *ced-3/ICE/nedd-2* gene family function in programmed cell death in a wide variety of species.

mIch-2

The cDNA sequence and deduced amino acid sequence of *mIch-2* are shown in Figure 6. As expected, *mIch-2* shows homology to both *hICE* and *mICE* as well as to *C. elegans ced-3* (see Figure 7 and 7A). In contrast to other cell-death genes that have been identified, *mIch-2* is preferentially expressed in the thymus and placenta. Example 3 describes how the gene was obtained by screening a mouse thymus cDNA library with a DNA probe derived from *hICE* under conditions of low stringency. Given the amino acid sequence and cDNA sequence shown in Figure 6, preferred methods of obtaining the *mIch-2* gene (either genomic or cDNA) are described below.

Ich-1

nedd2, *hICE*, *mIch-2* and *ced-3* are all members of the same gene family. This suggested that new genes might be isolated based upon their homology to identified family members.

Ich-1 is 1456 base pairs in length and contains an open reading frame of 435 amino acids (Figure 10A). The C-terminal 130 amino acids of Ich-1 are over 87% identical to mouse nedd2. However, Ich-1 contains a much longer open reading frame and has the pentapeptide QACRG which is the active center of the proteins of the *ced-3/ICE* family. The results indicate that the cDNA isolated by Kumar *et al.* may not have been synthesized from a fully processed mRNA and that the 5' 1147 base pairs which Kumar *et al.* reported for *nedd2* cDNA may actually represent the sequence of an intron. The sequence reported by Kumar *et al.* contains a region which could potentially code for QACRG but these amino acids are encoded in a different reading frame than that indicated by Kumar *et al.* (Figure 8). This suggests that Kumar *et al.* made an error in cloning.

The coding regions of nedd2 and Ich-1_L are highly homologous. The amino acid sequence of the deduced Ich-1_L protein shares 28% identity with ced-3 and 27% identity with hICE (Figures 9, 9A).

Ich-1 mRNA is alternatively spliced into two different forms. One mRNA species encodes a protein product of 435 amino acids, designated Ich-1_L, which contains amino acid sequence homologous to both P20 and P10 subunits of hICE as well as entire ced-3 (28% identity). The other mRNA encodes a 312 amino-acid truncated version of Ich-1, designated Ich-1_S, that terminates 21 amino acid residues after the QACRG active domain of Ich-1. Expression of *Ich-*1_L and *Ich-*1_S has opposite effects on cell death. Overexpression of *Ich-*1_L induces Rat-1 fibroblast cells to die in culture, while overexpression of the *Ich-*1_S suppresses Rat-1 cell death induced by serum deprivation. Results herein suggest that *Ich-1* may play an important role in both positive and negative regulation of programmed cell death in vertebrate animals.

Ich-3

Ich-3 was identified based on its sequence homology with *hICE* and other isolated *ICE* homologs. Since the *Ich-3* clone isolated by PCR only contains the coding region for the C-terminal half of Ich-3, a mouse thymus cDNA library was screened using the *Ich-3* insert. Among 2 million clones screened, 9 positive clones were isolated. The sequence herein is from one clone that contains the complete coding region for *Ich-3* gene.

Methods of Making

ced-3

There are many standard procedures for cloning genes which are well-known in the art and which can be used to obtain the *ced-3* gene (see e.g.,

Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, 2nd edition, vol. 1-3, Cold Spring Harbor Laboratory Press, 1989). In Example 1, a detailed description is provided of two preferred procedures. The first preferred procedure does not require the availability of *ced-3* gene sequence information and is based upon a method described by Ruvkun *et al.* (*Molecular Genetics of Caenorhabditis Elegans Heterochromic Gene lin-14 121*: 501-516 (1988)). In brief, Bristol and Bergerac strains of nematode are crossed and restriction fragment length polymorphism mapping is performed on the DNA of the resulting inbred strain. Restriction fragments closely linked to *ced-3* are identified and then used as probes to screen cosmid libraries for cosmids carrying all or part of the *ced-3* gene. Positive cosmids are injected into a nematode strain in which *ced-3* has been mutated. Cosmids carrying active *ced-3* genes are identified by their ability to rescue the *ced-3* mutant phenotype

A second method for cloning *ced-3* genes relies upon the sequence information which has been disclosed herein. Specifically, DNA probes are constructed based upon the sequence of the *ced-3* gene of *C. elegans*. These probes are labelled and used to screen DNA libraries from nematodes or other species. Procedures for carrying out such cloning and screening are described more fully below in connection with the cloning and expression of *mIch-2*, *Ich-1*, and *Ich-3*, and are well-known in the art (see, e.g., Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, 2nd edition (1988)). When hybridizations are carried out under conditions of high stringency, genes are identified which contain sequences corresponding exactly to that of the probe. In this way, the exact same sequence as described by the inventors herein may be obtained. Alternatively, hybridizations may be carried out under conditions of low stringency in order to identify genes in other species which are homologous to *ced-3* but which contain structural variations (see Example 1 for a description of how such hybridizations may be used to obtain the *ced-3* genes from *C. briggsae* and *C. vulgaris*).

The results in Example 2 demonstrate that the products of cell-death genes may be tolerated by cells provided they are expressed at low levels.

Therefore, ced-3 may be obtained by incorporating the *ced-3* cDNA described above into any of a number of expression vectors well-known in the art and transferring these vectors into appropriate hosts (see Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, vol. 3 (1988)). As described below in connection with the expression of *mIch-2*, *Ich-1*, and *Ich-3*, expression systems may be utilized in which cells are grown under conditions in which a recombinant gene is not expressed and, after cells reach a desired density, expression may be induced. In this way, the tendency of cells which express *ced-3* to die may be circumvented.

mIch-2, Ich-1, and Ich-3

DNA encoding mIch-2, Ich-1, and Ich-3 may be obtained from either genomic DNA or from cDNA. Genomic DNA may include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with the 5′ promoter region of the sequences and/or with the 3′ transcriptional termination region. Further, such genomic DNA may be obtained in association with the genetic sequences which encode the 5′ non-translated region of the *mIch-2*, *Ich-1*, and *Ich-3* mRNA and/or with the genetic sequences which encode the 3′ non-translated region. To the extent that a host cell can recognize the transcriptional and/or translational regulatory signals associated with the expression of the mRNA and protein, then the 5′ and/or 3′ non-transcribed regions of the native gene, and/or, the 5′ and/or 3′ non-translated regions of the mRNA, may be retained and employed for transcriptional and translational regulation.

Genomic DNA can be extracted and purified from any cell containing mouse chromosomes by means well known in the art (for example, see *Guide to Molecular Cloning Techniques*, S.L. Berger *et al.*, eds., Academic Press (1987)). Alternatively, mRNA can be isolated from any cell which expresses the genes, and used to produce cDNA by means well known in the art (*Id.*). The preferred sources for *mIch-2* are thymus or placental cells. The mRNA

coding for any of the proteins may be enriched by techniques commonly used to enrich mRNA preparations for specific sequences, such as sucrose gradient centrifugation, or both.

For cloning into a vector, DNA prepared as described above (either human genomic DNA or preferably cDNA) is randomly sheared or enzymatically cleaved, and ligated into appropriate vectors to form a recombinant gene library. A DNA sequence encoding the protein or its functional derivatives may be inserted into a DNA vector in accordance with conventional techniques. Techniques for such manipulations are disclosed by Sambrook, *et al.*, *supra*, and are well known in the art.

In a preferred method, oligonucleotide probes specific for the gene are designed from the cDNA sequences shown in the Figures 6, 10A, 10B, and 14. The oligonucleotide may be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., Academic Press, San Diego, CA (1987)) and employed as a probe to identify and isolate the cloned gene by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Maniatis, T., *et al.* (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)), and by Hames, B.D., *et al.* (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)). Those members of the above-described gene library which are found to be capable of such hybridization are then analyzed to determine the extent and nature of the coding sequences which they contain.

To facilitate the detection of the desired coding sequence, the above-described DNA probe is labeled with a detectable group. This group can be any material having a detectable physical or chemical property. Such materials are well-known in the field of nucleic acid hybridization and any label useful in such methods can be applied to the present invention. Particularly useful are radioactive labels, such as ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or the like. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. The oligonucleotide may be radioactively

labeled, for example, by "nick-translation" by well-known means, as described in, for example, Rigby, P.J.W., *et al.*, *J. Mol. Biol. 113*:237 (1977) or by T4 DNA polymerase replacement synthesis as described in, for example, Deen, K.C., *et al.*, *Anal. Biochem. 135*:456 (1983).

Alternatively, oligonucleotide probes may be labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., *et al.*, *Proc. Natl. Acad. Sci. USA 80*:4045 (1983); Renz, M., *et al.*, *Nucl. Acids Res. 12*:3435 (1984); and Renz, M., *EMBO J. 6*:817 (1983).

For *Ich-1*, the isolation shown in the Examples was as follows. Two primers were used in the polymerase chain reaction to amplify *nedd2* cDNA from embryonic day 15 mouse brain cDNA (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, vol. 3 (1988)). One primer had the sequence: ATGCTAACTGTCCAAGTCTA and the other primer had the sequence: TCCAACAGCAGGAATAGCA. The cDNA thus amplified was cloned using standard methodology. The cloned mouse *nedd2* cDNA was used as a probe to screen a human fetal brain cDNA library purchased from Stratagene. Such methods of screening and isolating clones are well known in the art (Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)); Hames, B.D., *et al.*, *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)). A human *nedd*-2 cDNA clone was isolated that encodes a protein much longer than the mouse nedd-2 and contains amino acid sequences homologous to the entire hICE and ced-3. The isolated clone was given the name *Ice-ced 3* homolog or *Ich-1*.

The *Ich-1* cDNA may be obtained using the nucleic acid sequence information given in Figures 10A or 10B. DNA probes constructed from this sequence can be labeled and used to screen human gene libraries as described herein. Also as discussed herein, *Ich-1* may be cloned into expression vectors and expressed in systems in which host cells are grown under conditions in which recombinant genes are not expressed and, after cells reach a desired

density, expression is induced. In this way, a tendency of cells which express *Ich-1* to die may be circumvented.

One method of making *Ich-3* is as follows. mRNA was isolated from embryonic day 14 mouse embryos using Invitrogen's microfast track mRNA isolation kit. The isolated mRNA was reverse transcribed to generate template for PCR amplification. The degenerate PCR primers were: *cICEB* {TG(ATCG)CC(ATCG)GGGAA(ATCG)AGGTAGAA} and *cICEAs* {ATCAT(ATC)ATCCAGGC(ATCG)TGCAG(AG)GG}. The PCR cycles were set up as follows: 1. 94°C, 3 min; 2. 94°C, 1 min; 3. 48°C, 2 min; 4. 72°C, 3 min; 5. return to "2" 4 cycles; 6. 94°C, 1 min; 7. 55°C, 2 min; 8. 72°C, 3 min; 9. return to "6" 34 cycles; 10. 72°C, 10 min; 11. end. Such PCR generated a band about 400bp, the predicted size of *ICE* homologs. The PCR products were cloned into T-tailed blunt-ended pBSKII plasmid vector (Stratagene). Plasmids that contain an insert were analyzed by DNA sequencing.

The *Ich-3* cDNA may also be obtained using the nucleic acid sequence information given in Figure 14. DNA probes constructed from this sequence can be labeled and used to screen human gene libraries as described herein. Also as discussed herein, *Ich-3* may be cloned into expression vectors and expressed in systems in which host cells are grown under conditions in which recombinant genes are not expressed and, after cells reach a desired density, expression is induced.

The methods discussed herein are capable of identifying genetic sequences which encode mIch-2, Ich-1, and Ich-3. In order to further characterize such genetic sequences, and, in order to produce the recombinant protein, it is desirable to express the proteins which these sequences encode.

To express any of the genes herein or their derivatives, transcriptional and translational signals recognizable by an appropriate host are necessary.

The cloned coding sequences, obtained through the methods described herein, may be operably linked to sequences controlling transcriptional expression in an expression vector and introduced into a host cell, either prokaryote or eukaryote, to produce recombinant protein or a functional derivative thereof. Depending upon which strand of the sequence is operably linked to the sequences controlling transcriptional expression, it is also possible to express antisense RNA or a functional derivative thereof.

Expression of the protein in different hosts may result in different post-translational modifications which may alter the properties of the protein. Preferably, the present invention encompasses the expression of *mIch-2*, *Ich-1*, and *Ich-3* or a functional derivative thereof, in eukaryotic cells, and especially mammalian, insect and yeast cells. Especially preferred eukaryotic hosts are mammalian cells either *in vivo*, or in tissue culture. Mammalian cells provide post-translational modifications which should be similar or identical to those found in the native protein. Preferred mammalian host cells include rat-1 fibroblasts, mouse bone marrow derived mast cells, mouse mast cells immortalized with Kirsten sarcoma virus, or normal mouse mast cells that have been co-cultured with mouse fibroblasts (Razin *et al.*, *J. of Immun. 132*:1479 (1984); Levi-Schaffer *et al.*, *Proc. Natl. Acad. Sci. (USA) 83*:6485 (1986) and Reynolds *et al.*, *J. Biol. Chem. 263*:12783-12791 (1988)).

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the nucleotide sequence which encodes the polypeptide.

An operable linkage is a linkage in which a coding sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the coding sequence under the influence or control of the regulatory sequence. Two DNA sequences (e.g. the coding sequence of protein and a promoter) are said to be operably linked if induction of promoter function results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the

introduction of a frame-shift mutation; (2) interfere with the ability of regulatory sequences to direct the expression of the coding sequence, antisense RNA, or protein; or (3) interfere with the ability of the coding sequence template to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

The precise nature of the regulatory regions needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5′ non-transcribing and 5′ non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5′ non-transcribing control sequences will include a region which contains a promoter for transcriptional control of the operably linked gene.

Expression of proteins of the invention in eukaryotic hosts requires the use of regulatory regions functional in such hosts, and preferably eukaryotic regulatory systems. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the eukaryotic host. The transcriptional and translational regulatory signals can also be derived from the genomic sequences of viruses which infect eukaryotic cells, such as adenovirus, bovine papilloma virus, simian virus, herpesvirus, or the like. Preferably, these regulatory signals are associated with a particular gene which is capable of a high level of expression in the host cell.

In eukaryotes, where transcription is not linked to translation, control regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell. Promoters from heterologous mammalian genes which encode mRNA capable of translation are preferred, and especially, strong promoters such as the promoter for actin, collagen, myosin, etc., can be employed provided they also function as promoters in the host cell. Preferred eukaryotic promoters include the promoter of the mouse

metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of herpesvirus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature (London) 290*:304-310 (1981)); in yeast, the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)) or a glycolytic gene promoter may be used.

It is known that translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the proteins of the invention or functional derivatives thereof, does not contain any intervening codons which are capable of encoding a methionine. The presence of such codons results either in the formation of a fusion protein or a frame-shift mutation.

If desired, a fusion product of the proteins may be constructed. For example, the sequence coding for the proteins may be linked to a signal sequence which will allow secretion of the protein from, or the compartmentalization of the protein in, a particular host. Such signal sequences may be designed with or without specific protease sites such that the signal peptide sequence is amenable to subsequent removal. Alternatively, the native signal sequence for this protein may be used.

Transcriptional initiation regulatory signals can be selected which allow for repression or activation, so that expression of operably linked genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

If desired, the non-transcribed and/or non-translated regions 3′ to the sequence coding for the proteins can be obtained by the above-described cloning methods. The 3′-non-transcribed region may be retained for transcriptional termination regulatory sequence elements; the 3′-non-translated region may be retained for translational termination regulatory sequence

elements, or for those elements which direct polyadenylation in eukaryotic cells. Where native expression control signals do not function satisfactorily in a host cell, functional sequences may be substituted.

The vectors of the invention may further comprise other operably linked regulatory elements such as enhancer sequences, or DNA elements which confer tissue or cell-type specific expression on an operably linked gene.

To transform a mammalian cell with the DNA constructs of the invention many vector systems are available, depending upon whether it is desired to insert the DNA construct into the host cell chromosomal DNA, or to allow it to exist in extrachromosomal form. If the protein encoding sequence and an operably linked promoter are introduced into a recipient eukaryotic cell as a non-replicating DNA (or RNA) molecule, the expression of the protein may occur through the transient expression of the introduced sequence.

In a preferred embodiment, genetically stable transformants may be constructed with vector systems, or transformation systems, whereby *mIch-2*, *Ich-1*, or *Ich-3* DNA is integrated into the host chromosome. Such integration may occur *de novo* within the cell or, in a most preferred embodiment, through the aid of a cotransformed vector which functionally inserts itself into the host chromosome, for example, retroviral vectors, transposons or other DNA elements which promote integration of DNA sequences in chromosomes.

Cells which have stably integrated the introduced DNA into their chromosomes are selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector in the chromosome, for example the marker may provide biocide resistance, e.g., resistance to antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

In another embodiment, the introduced sequence is incorporated into a plasmid or viral vector capable of autonomous replication in the recipient

host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred eukaryotic plasmids include those derived from the bovine papilloma virus, vaccinia virus, SV40, and, in yeast, plasmids containing the 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., *et al.*, *Miami Wntr. Symp. 19*:265-274 (1982); Broach, J.R., In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, J.R., *Cell 28*:203-204 (1982); Bollon, D.P., *et al.*, *J. Clin. Hematol. Oncol. 10*:39-48 (1980); Maniatis, T., In: *Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Expression*, Academic Press, NY, pp. 563-608 (1980)), and are commercially available.

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable means, including transfection. After the introduction of the vector, recipient cells are grown in a medium which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the protein, or in the production of a fragment of this protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner, for example, expression which follows induction of differentiation of the transformed cells (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). The latter is preferred for the expression of the proteins of the invention. By growing cells under conditions in which the proteins are not expressed, cell death may be avoided. When a high cell

density is reached, expression of the proteins may be induced and the recombinant protein harvested immediately before death occurs.

The expressed protein is isolated and purified in accordance with conventional procedures, such as extraction, precipitation, gel filtration chromatography, affinity chromatography, electrophoresis, or the like.

The *mIch-2*, *Ich-1*, and *Ich-3* sequences, obtained through the methods above, will provide sequences which not only encode proteins but which also provide for transcription of *mIch-2*, *Ich-1*, and *Ich-3* antisense RNA; the antisense DNA sequence will be that sequence found on the opposite strand of the strand transcribing the mRNA. The antisense DNA strand may also be operably linked to a promoter in an expression vector such that transformation with this vector results in a host capable of expression of the antisense RNA in the transformed cell. Antisense DNA and RNA may be used to interact with endogenous *mIch-2*, *Ich-1*, or *Ich-3* DNA or RNA in a manner which inhibits or represses transcription or translation of the genes in a highly specific manner. Use of antisense nucleic acid to block gene expression is discussed in Lichtenstein, C., *Nature 333*:801-802 (1988).

Methods of Using

ced-3

The *ced-3* gene (as well as *ced-3* homologs and other members of the *ced-3* gene family) may be used for a number of distinct purposes. First, portions of the gene may be used as a probe for identifying genes homologous to *ced-3* in other strains of nematode (see Example 1) as well as in other species (see Examples 2 and 3). Such probes may also be used to determine whether the *ced-3* gene or homologs of *ced-3* are being expressed in cells.

The cell death genes will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. Among diseases and conditions which could potentially be treated are neural and

muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging. The discovery that *ced-3* is related to *ICE* suggests that cell death genes may play an important role in inflammation (IL-1β is known to be involved in the inflammatory response). Thus therapeutics based upon *ced-3* and related cell death genes may also be developed.

mIch-2, Ich-1, and Ich-3

mIch-2, *Ich-1*, and *Ich-3* will have the same uses as those described in connection with *ced-3* (above) and *ICE* (see below). The gene sequences may be used to construct antisense DNA and RNA oligonucleotides, which, in turn, may be used to prevent programmed cell death in thymus or placental cells. Techniques for inhibiting the expression of genes using antisense DNA or RNA are well-known in the art (Lichtenstein, C., *Nature 333*:801-802 (1988)). Portions of the claimed DNA sequence may also be used as probes for determining the level of expression. Similarly the protein may be used to generate antibodies that can be used in assaying cellular expression.

Portions of the *mIch-2*, *Ich-1*, and *Ich-3* genes described above may be used for determining the level of expression of the proteins (*mIch-2* in thymus or placental cells as well as in other tissues and organs). Such methods may be useful in determining if these cells have undergone a neoplastic transformation. Probes based upon the gene sequences may be used to isolate similar genes involved in cell death. A portion of the gene may be used in homologous recombination experiments to repair defective genes in cells or, alternatively, to develop strains of mice that are deficient in the gene. Antisense constructs may be transfected into cells, according to the native cellular expression patterns of each gene (placental or thymus cells for *mIch-2*, for example) in order to develop cells which may be maintained in culture for an extended period of time or indefinitely. Alternatively antisense constructs may be used in cell culture or *in vivo* to block cell death.

The protein may be used for the purpose of generating polyclonal or monoclonal antibodies using standard techniques well known in the art (Klein, J., *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, N.Y. (1982); Kennett *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, N.Y. (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology 13*, Burdon *et al.* eds., Elseiver, Amsterdam (1984); Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988)). Such antibodies may be used in assays for determining the expression of the genes. Purified protein would serve as the standard in such assays.

Based upon the sequences of Figures 6, probes may be used to determine whether the *mIch-2* gene or homologs of *mIch-2* are being expressed in cells. Such probes may be utilized in assays for correlating *mIch-2* expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *mIch-2*.

mIch-2 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequences shown in Figure 6 may be used to inhibit *mIch-2* expression. Such inhibition will be useful in blocking cell death in cultured cells.

mIch-2 may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (see above). The antibodies may be used in assays for determining the expression of *mIch-2*. Purified mIch-2 would serve as the standard in such assays.

Based upon the sequences of Figures 10A and 10B, probes may be used to determine whether the *Ich-1* gene or homologs of *Ich-1* are being expressed in cells. Such probes may be utilized in assays for correlating *Ich-1*

expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *Ich-1*.

Ich-1 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequences shown in Figures 10A and 10B, may be used to inhibit *Ich-1* expression. Such inhibition will be useful in blocking cell death in cultured cells.

Ich-1 protein may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (see above). The antibodies may be used in assays for determining the expression of *Ich-1*. Purified Ich-1 would serve as the standard in such assays.

Based upon the sequence of Figure 14, probes may be used to determine whether the *Ich-3* gene or homologs of *Ich-3* are being expressed in cells. Such probes may be utilized in assays for correlating *Ich-3* expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *Ich-3*.

Ich-3 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequence shown in Figure 14 may be used to inhibit *Ich-3* expression. Such inhibition will be useful in blocking cell death in cultured cells.

Ich-3 protein may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (see above). The antibodies may be used in assays for determining the expression of *Ich-3*. Purified *Ich-3* would serve as the standard in such assays.

Method for Preventing Programmed Cell Death in Vertebrate Cells by Inhibiting the Enzymatic Activity of ICE

The present invention is directed to preventing the programmed death of vertebrate cells by inhibiting the action of *ICE*. The detailed structural analysis performed on the *ced-3* gene from *C. elegans* revealed a homology to human and murine *ICE* which is especially strong at the QACRG active domain of *hICE* (see Figure 3A).

In order to determine if *ICE* functions as a cell death gene in vertebrates, the *mICE* gene was cloned, inserted into an expression vector and then transfected into rat cells. A close correlation was found between *ICE* expression and cell death (see Example 2).

Further support for the function of *ICE* as a cell death gene was obtained from inhibition studies. In order to determine whether cell death can be prevented by inhibiting the enzymatic action of *ICE*, cell lines were established which produced a high level of crmA. When these cells were transfected with *mICE*, it was found that a large percentage of the cells expressing *mICE* maintained a healthy morphology and did not undergo programmed cell death (Example 2 herein).

Evidence that *ICE* has a physiological role as a vertebrate cell death gene was also obtained by examining cells engineered to over-express *bcl-2*, an oncogene known to inhibit programmed cell death and to be overexpressed in many follicular and B cell lymphomas. It was found that cells expressing *bcl-2* did not undergo cell death despite the high levels of *ICE* expression (Example 2 herein). These results suggest that *bcl-2* may promote malignancy by inhibiting the action of *ICE*.

Any method of specifically regulating the action of *ICE* in order to control programmed cell death in vertebrates is encompassed by the present invention. This would include using not only inhibitors specific to ICE, e.g. crmA, or the inhibitors described by Thornberry *et al.* (*Nature 356*:768-774 (1992)), but also any method which specifically prevented the expression of

the *ICE* gene. Thus, antisense RNA or DNA comprised of nucleotide sequences complementary to *ICE* and capable of inhibiting the transcription or translation of *ICE* are within the scope of the invention (see Lichtenstein, C., *Nature 333*:801-802 (1988)).

The ability to prevent vertebrate programmed cell death is of use in developing cells which can be maintained for an indefinite period of time in culture. For example, cells over-expressing *crmA* may be used as hosts for expressing recombinant proteins. The ability to prevent programmed cell death may allow cells to live independent of normally required growth factors. It has been found that microinjecting *crmA* mRNA or a *crmA*-expressing nucleic acid construct into cells allows chicken sympathetic neurons to live *in vitro* after the removal of neural growth factor.

Method for Promoting Programmed Cell Death in Vertebrate Cells by Increasing or Inducing the Activity of ICE

The expression of *ICE* can be increased in order to cause programmed cell death. For example, homologous recombination can be used to replace a defective region of an *ICE* gene with its normal counterpart. The level of regulation amenable to manipulation to either increase or decrease the expression of *ICE* include DNA, RNA, or protein. Genomic DNA, for example, can be mutated by the introduction of selected DNA sequences introduced into the genome by homologous recombination. Any desired mutation can be introduced *in vitro* and, through gene replacement, either decoding or regulatory sequences of the gene can be manipulated. Extrachromosomal DNA with the appropriate gene sequence can also be introduced into cells to compete with the endogenous product. At the level of RNA, antisense RNA molecules can be introduced, as well as RNA having more or less affinity for the translational apparatus or greater or lesser tendency to be transcribed. At the level of protein, protein counterparts can be designed having a higher or lower activity.

In addition to direct regulation, and particularly an increase in gene expression of *ICE*, the possibility also exists for indirect regulation by regulating those cellular components that either induce or suppress the expression of *ICE*. The inventors have found, for example, that TNF-α induces a program of cell death via the activation of *hICE* genetic sequences. Thus, a further level of regulation is that of modulating the expression of TNF-α and its functional counterparts. Thus, any cellular component regulating programmed cell death by means of the *ICE/ced-3* pathway can itself be regulated rather than directly regulating the *ICE* gene. Regulation can occur by any of the means discussed above, for example. The genes in the *bcl-2* family, p53 and the genes that are regulated by p53 (such as p21), the proteins in the *ras* pathway (*ras*, *raf* 14-3-3), Fas and the proteins in the cytotoxic T cell granules (such as granzyme B) may all directly and indirectly influence the activity of the *ICE* family. Accordingly, the regulation can also occur by means of any of these genes and others in such pathways. In this way, it may be possible to prevent the uncontrolled growth of certain malignant cells.

Methods of increasing *ICE* activity may be used to kill undesired organisms such as parasites. crmA is a viral protein that is important for cowpox infection. This suggests that the prevention of cell death may be important for successful infection and that the promotion of *ICE* expression may provide a means for blocking infection. Activation of *ICE* family genes may be used to eliminate cancerous cells or any other unwanted cells. Prevention of cell death by inactivating the *ICE* family of genes could prevent neuronal degenerative diseases, such as Alzheimer's disease, amylotrophic lateral sclerosis, and cell death associated with stroke, ischemic heart injury, and aging.

Having now generally described this invention, the same will be further described by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless

otherwise specified. All references cited throughout the specification are incorporated by reference in their entirety.

Example 1

Experimental Procedures

General methods and strains

The techniques used for culturing *C. elegans* have been described by Brenner (Brenner, S., *Genetics 77*:71-94 (1974)). All strains were grown at 20°C. The wild-type parent strains were *C. elegans* variety Bristol strain N2, Bergerac strain EM1002 (Emmons *et al., Cell 32*:55-65 (1983)), *C. briggsae* and *C. vulgaris*. The genetic markers used are described below. These markers have been previously described (Brenner, S., *Genetics 77*:71-94 (1974)); and Hodgkin *et al., Genetics in the Nematode Caenorhabditis Elgens* (Wood *et al.* eds.) pp.491-584, Cold Spring Harbor, New York (1988)). Genetic nomenclature follows the standard system (Horvitz *et al., Mol. Gen. Genet. 175*:129-133 (1979)).

LG I: *ced-1 (ei 735); unc-54 (r323)*

LG VI: *unc-31 (e928), unc-30 (e191), ced-3 (n717, n718, n1040, n1129, n11634, n1164, n1165, n1286, n1949, n2426, n2430, n2433), unc-26 (e205), dpy-4 (e1166)*

LG V: *eg-1(n986); unc-76 (e911)*

LG X: *dpy-3(e27)*

Isolation of additional alleles of ced-3

A non-complementation screen was designed to isolate new alleles of *ced-3*. Because animals heterozygous for *ced3(n717) in trans* to a deficiency are viable (Ellis *et al., Cell 44*:817-829 (1986)), it was expected that animals

carrying a complete loss-of-function mutant *ced-3* allele *in trans* to *ced-3(n717)* would be viable even if homozygotes for the allele were inviable. EMS mutagenized *egl-1* L4 males were mated with *ced-3(n717) unc-26(e205)*; *egl-1(n487)*; *dpy-3(e27)* hermaphrodites. *egl-1* was used as a marker in this screen. Dominant mutations in *egl-1* cause the two hermaphrodite-specific neurons, the HSNs, to undergo programmed cell death (Trent *et al., Genetics 104*:619-647 (1983)). The HSNs are required for normal egg-laying, and *egl-1(n986)* hermaphrodites, which lack HSNs are egg-laying defective (Trent *et al., Genetics 104*:619-647)). The mutant phenotype of *egl-1* is suppressed in a *ced-3*; *egl-1* strain because mutations in *ced-3* block programmed cell deaths. *egl-1* males were mutagenized with EMS and crossed with *ced3(n717) unc-26(e205)*; *egl-1(n487)*; *dpy-3(e27)*. Most cross progeny were egg-laying defective because they were heterozygous for *ced-3* and homozygous for *egl-1*. Rare egglaying competent animals were picked, those animals being candidates for carrying new alleles of *ced-3*. Four such animals were isolated from about 10,000 F1 cross progeny of EMS-mutagenized animals. These new mutations were made homozygous to confirm that they carried mutations of *ced-3*.

RFLP mapping

Two cosmid libraries were used extensively in this work - a *Sau3A* I partial digest genomic library of 7000 clones in the vector pHC79 and a *Sau3A* I partial digest genomic library of 6000 clones in the vector pJB8 (Coulson *et al., Proc. Natl. Acad. Sci. U.S.A. 83*:7821-7825 (1986).

Bristol (N2) and Bergerac (EM1002) DNA was digested with various restriction enzymes and probed with different cosmids to look for RFLPs. *nP33* is a *Hind*III RFLP detected by the "right" end of Jc8. The "right" end of Jc8 was made by digesting Jc8 with *Eco*RI and self-ligating. *nP34* is a *Hind*III RFLP detected by the "left" end of Jc8. The "left" end of Jc8 was made by digesting Jc8 by *Sal*I and self ligating. *nP36* and *nP37* are both *Hind*III RFLPs detected by T10H5 and B0564, respectively.

Germ line transformation

The procedure used for microinjection basically follows that of A. Fire (Fire, A., *EMBO J. 5*:2673-2680 (1986)). Cosmid DNA was twice CsCl gradient purified. Miniprep DNA was used when deleted cosmids were injected and was prepared from 1.5 ml overnight bacterial culture in superbroth. Superbroth was prepared by combining 12 g Bacto tryptone, 24 g yeast extract, 8 ml 50% glycerol and 900 ml H_2O. The mixture was autoclaved and then 100 ml of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 were added. The bacterial culture was extracted by the alkaline lysis method as described in Maniatis *et al.* (*Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1983)). DNA was treated with RNase A (37°C, 30 min) and then with protease K (55°C, 30 min). The preparation was phenol- and then chloroform-extracted, precipitated twice (first in 0.3 M Na acetate and second in 0.1 M K acetate, pH 7.2), and resuspended in 5 l injection buffer as described by A. Fire (Fire, A., *EMBO J. 5*:2673-2680 (1986)). The DNA concentration for injection was in the range of 100 μg to 1 mg per ml.

All transformation experiments used the *ced-1(e1735)*; *unc-31(e928) ced-3(n717)* strain. *unc-31* was used as a marker for co-transformation (Kim *et al.*, *Genes & Dev. 4*:357-371 (1990)). *ced-1* was present to facilitate scoring of the ced-3 phenotype. The mutations in *ced-1* block the engulfment process of cell death, which makes the corpses of the dead cells persist much longer than that in the wild-type (Hedgecock *et al.*, *Science 220*:1277-1280 (1983)). ced-3 phenotype was scored as the number of dead cells present in the head of young L1 animals. The cosmid C10D8 or the plasmid subclones of C10D8 were mixed with C14G10 (*unc-31(+)*-containing) at a ratio of 2:1 or 3:1 to increase the chances that an Unc-31(+) transformant would contain the cosmid or plasmid being tested. Usually, 20-30 animals were injected in one experiment. Non-*Unc* F1 progeny of injected animals were isolated three to four days later. About 1/2 to 1/3 of the non-*Unc* progeny transmitted the

non-*Unc* phenotype to F2 and established a line of transformants. The young L1 progeny of such non-Unc transformants were checked for the number of dead cells present in the head using Nomarski optics.

Determination of ced-3 transcript initiation site

Two primers,
Pex1: (5'GTTGCACTGCTTTCACGATCTCCCGTCTCT3')
and Pex2: (5'TCATCGACTTTTAGATGACTAGAGAACATC3'), were used for primer extension. The primers for RT-PCR were:
SL1 (5'GTTTAATTACCCAAGTTTGAG3')
and log-5 (5'CCGGTGACATTGGACACTC3'). The products were reamplified using the primers SL1 and oligo10 (5'ACTATTCAACACTTG3'). A product of the expected length was cloned into the PCR1000 vector (Invitrogen) and sequenced.

Determination and analysis of DNA sequence

For DNA sequencing, serial deletions were made according to a procedure developed by Henikoff (Heinkoff, S., *Gene 28*:351-359 (1984)). DNA sequences were determined using Sequenase and protocols obtained from US Biochemicals with minor modifications.

The ced-3 amino acid sequence was compared with amino acid sequences in the GenBank, PIR and SWISS-PROT databases at the National Center for Biotechnology Information (NCBI) using the *blast* network service.

Cloning of ced-3 genes from other nematode species

C. briggsae and *C. vulgaris ced-3* genes were isolated from corresponding phage genomic libraries using the *ced-3* cDNA subclone pJ118 insert as a probe under low stringency conditions (5xSSPE, 20% formamide,

0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone, and 1% SDS) at 40°C overnight and washed in 1xSSPE and 0.5% SDS twice at room temperature and twice at 42°C for 20 min each time.

Results

ced-3 is not essential for viability

All previously described *ced-3* alleles were isolated in screens designed to detect viable mutants in which programmed cell death did not occur (Ellis *et al.*, *Cell 44*:817-829 (1986)). Such screens might systematically have missed classes of *ced-3* mutations that result in inviability. Since animals with the genotype of *ced-3*/deficiency are viable (Ellis *et al.*, *Cell 44*:817-829 (1986)), a noncomplementation-screening scheme was designed that would allow the isolation of recessive lethal alleles of *ced-3*. Four new *ced-3* alleles (*n1163*, *n1164*, *n1165*, and *n1286*) were obtained which were viable as homozygotes. These new alleles were isolated at a frequency of about 1 in 2500 mutagenized haploid genomes, approximately the frequency expected for the generation of null mutations in an average *C. elegans* gene (Brenner, S., *Genetics 77*:71-94 (1974); Meneely *et al.*, *Genetics 92*:99-105 (1990); Greenwald *et al.*, *Genetics 96*:147-160 (1980)).

These results suggest that animals lacking *ced-3* gene activity are viable. In support of this hypothesis, molecular analysis has revealed that three *ced-3* mutations are nonsense mutations that prematurely terminate *ced-3* translation and one alters a highly conserved splice acceptor site (see below). These mutations would be expected to eliminate *ced-3* activity completely. Based upon these considerations, it was concluded that *ced-3* gene activity is not essential for viability.

ced-3 is contained within a 7.5 kb genomic fragment

The *ced-3* gene was cloned using the approach of Ruvkun *et al.* (*Molecular Genetics of the Caenorhabditis elegans Heterochronic Gene lin-14* *121*:501-516 (1988)). Briefly (for further details, see Experimental Procedures), the *C. elegans* Bristol strain N2 contains 30 dispersed copies of the transposable element Tc1, whereas the Bergerac strain contains more than 400 copies (Emmons *et al.*, *Cell 32*:55-65 (1983); Finney, M., Ph.D. Thesis, "The Genetics and Molecular Biology of *unc-86*, a *Caenorhabditis elegans* Cell Lineage Gene," Cambridge, MA (1987)). By crossing Bristol and Bergerac strains, a series of recombinant inbred strains were generated in which chromosomal material was mostly derived from the Bristol strain with varying amounts of Bergerac-specific chromosome IV-derived material in the region of the *ced-3* gene. By probing DNA from these strains with plasmid pCe2001 which contains Tc1 (Emmons *et al.*, *Cell 32*:55-65 (1983), a 5.1 kb EcoRI Tc1-containing restriction fragment specific to the Bristol strain (restriction fragment length polymorphism nP35) and closely linked to *ced-3* was identified.

Cosmids that contained this 5.1 kb restriction fragment were identified and it was found that these cosmids overlapped an existing cosmid contig that had been defined as part of the *C. elegans* genome project (Coulson *et al.*, *Proc. Natl. Acad. Sci. 83*:7821-7825 (1986). Four other Bristol-Bergerac restriction fragment length polymorphisms were defined by cosmids in this contig (*nP33, np34, nP36, nP37*). By mapping these restriction fragment length polymorphisms with respect to the genes *unc-30*, *ced-3* and *unc-26*, the physical contiguity was oriented with respect to the genetic map and the region containing the *ced-3* gene was narrowed to an interval spanned by three cosmids (Fig. 1).

On Southern blot, three of three + Berg *unc-26* recombinants showed the Bristol nP33 pattern while two of two *ced-3* + Berg recombinants showed the Bergerac pattern (data not shown). Thus, nP33 maps very close or to the

right of *unc-26*. For nP34, two of two *ced-3* + Berg recombinants and two of three + Berg *unc-26* recombinants showed the Bergerac pattern; one of the three + Berg *unc-26* recombinant showed the Bristol pattern (data not shown). The genetic distance between *ced-3* and *unc-26* is about 0.2 mu. Thus, nP34 maps between *ced-3* and *unc-26*, about 0.1 mu to the right of *ced-3*. Similar experiments mapped nP35, the 5.1 kb Bristol specific Tc1 element, to about 0.1 mu to the right of *ced-3*.

In order to map *n36* and *n37*, Bristol *unc-30 ced-3*/+ + males were crossed with Bergerac hermaphrodites. From the progeny of heterozygotes of genotype *unc-30 ced-3* (Bristol)/+ + (Bergerac), Unc-30/non-ced-3 and non-Unc-30/ced-3 animals were picked and DNA was prepared from these strains. *nP36* maps very close or to the right of *unc-30* since two of two *unc-30* + Berg recombinants showed Bristol pattern and two of two + Berg *ced-3* recombinants showed the Bergerac pattern (data not shown). Similarly, nP37 maps very close or to the right of *unc-30* since four of the four + Berg *ced-3* showed Bergerac pattern and six of six *unc-30* + Berg recombinants showed the Bristol pattern (data not shown). These experiments narrowed the region containing the ced-3 gene to an interval spanned by the three cosmids (Fig. 1a).

Cosmids that were candidates for containing the *ced-3* gene were microinjected (Fire, A., *EMBO J.* 5:2673-2680 (1986)) into *ced-3* mutant animals to test for rescue of the mutant phenotype. Specifically, cosmid C14G10, which contains the wild-type *unc-31* gene and a candidate cosmid were coinjected into *ced-1(e1375)*; *unc-31(e928) ced-3(n717)* hermaphrodites. Non-unc progeny were isolated and observed to see if the non-Unc phenotype was transmitted to the next generation, thus establishing a line of transformed animals. Young L1 progeny of such transformant lines were examined for the presence of cell deaths using Nomarski optics to see whether the ced-3 phenotype was complemented (see Experimental Procedures). Cosmid C14G10 alone does not confer *ced-3* activity when injected into a *ced-3* mutant.

unc-31 was used as a marker for co-transformation (Kim *et al.*, *Genes & Devel. 4*:357-371 (1990)). *ced-1* was present to facilitate scoring of the *ced-3* phenotype. Mutations in *ced-1* block the engulfment process of programmed cell death, causing the corpses of dead cells to persist much longer than in the wild-type (Hedgecock *et al.*, *Science 220*:1277-1280 (1983)). Thus, the presence of a corpse indicates a cell that has undergone programmed cell death. The *ced-3* phenotype was scored as the number of corpses present in the head of young L1 animals.

As indicated in Fig. 1, of the three cosmids injected (C43C9, W07H6 and C48D1), only C48D1 rescued the *ced-3* mutant phenotype. Both non-*Unc* transformed lines obtained, *nIs1* and *nEx2*, were rescued. Specifically, L1 *ced-1* animals contain an average of 23 cell corpses in the head, and L1 *ced-1*; *ced3* animals contain an average of 0.3 cell corpses in the head (Ellis *et al.*, *Cell 44*:817-829 (1986)). By contrast, *ced-1*; *unc-31 ced-3*; *nIs1*; and *ced-1*; *unc-31 ced-3*; *nEx2* animals contained an average of 16.4 and 14.5 cell corpses in the head, respectively. From these results, it was concluded that C48D1 contains the *ced-3* gene.

To locate *ced-3* more precisely within the cosmid C48D1, this cosmid was subcloned and the subclones tested for their ability to rescue the *ced-3* mutant phenotype (Fig. 1A). From these experiments, *ced-3* was localized to a DNA fragment of 7.5 kb (pJ7.5).

A 2.8 kb ced-3 transcript is expressed primarily during embryogenesis and independently of ced-4 function

The 7.6 kb pJ107 subclone of C48D1 (Fig. 1A) was used as a probe in a northern blot of polyA+ RNA derived from the wild-type *C. elegans* strain N2. This probe hybridized to a 2.8 kb transcript. Although this transcript is present in 11 different EMS-induced *ced-3* mutant strains, subsequent analysis has shown that all 11 tested *ced-3* mutant alleles contain

mutations in the genomic DNA that encodes this mRNA (see below), thus establishing this RNA as a *ced-3* transcript.

The developmental expression pattern of *ced-3* was determined by hybridizing a northern blot of RNA from animals at different stages of development with the *ced-3* cDNA subclone pJ118 (see below). The *ced-3* transcript was found to be most abundant during embryogenesis, when most programmed cell deaths occur, but was also detected during the L1 through L4 larval stages. It is present in relatively high levels in young adults.

Since *ced-3* and *ced-4* are both required for programmed cell death in *C. elegans*, and since both are highly expressed during embryonic development (Yuan *et al., Dev. 116*:309-320 (1992), the possibility existed that one of the genes might regulate the mRNA level of the other. Previous studies have revealed that *ced-3* does not regulate *ced-4* mRNA levels (Yuan *et al., Dev. 116*:309-320 (1992)). To determine if *ced-4* regulates *ced-3* mRNA levels, a northern blot of RNA prepared from *ced-4* mutant embryos was probed with the *ced-3* cDNA subclone pJ118. It was found that the amount and size of the *ced-3* transcript was normal in the *ced-4* mutants *n1162, n1416, n1894 and n1920.* Thus, *ced-4* does not appear to affect the steady-state levels of *ced-3* mRNA.

ced-3 cDNA and genomic Sequences

To isolate *ced-3* cDNA clones, *ced-3* genomic DNA pJ40 (Fig. 1A) was used as a probe to screen a cDNA library of the *C. elegans* wild-type strain N2 (Kim *et al., Genes & Dev. 4*:357-371 (1990)). The 2.5 kb cDNA clone pJ87 was isolated in this way. On northern blots, pJ87 hybridized to a 2.8 kb transcript and on Southern blots, it hybridized only to bands to which pJ40 hybridizes (data not shown). Thus, pJ87 represents an mRNA transcribed entirely from pJ40 which can rescue the *ced-3* mutant phenotype when microinjected into *ced-3* mutant animals. To confirm that pJ87 contains the *ced-3* cDNA, a frameshift mutation in the *SalI* site of pJ40 was made

corresponding to the *SalI* site in the pJ87 cDNA. Constructs containing the frameshift mutation failed to rescue the *ced-3* phenotype when microinjected into *ced-3* mutant animals (6 transformant lines; data not shown), suggesting that *ced-3* activity had been eliminated by mutagenizing a region of genomic DNA that corresponds to the pJ87 cDNA.

The DNA sequence of pJ87 is shown in Figure 2C. pJ87 contains an insert of 2482 bp with an open reading frame of 503 amino acids. It has 953 bp of 3′ untranslated sequence, not all of which is essential for *ced-3* expression; genomic constructs that do not contain 380 bp of the 3′-most region (pJ107 and its derivatives, see Fig. 1a) were capable of rescuing *ced-3* mutant phenotype. The cDNA ends with a poly-A sequence, suggesting that the complete 3′ end of the *ced-3* transcript is present.

To confirm the DNA sequence obtained from the *ced-3* cDNA and to study the structure of the *ced-3* gene, the genomic sequence of the *ced-3* gene from the plasmid pJ107 was determined. The insert in pJ107 is 7656 bp in length (Fig. 2).

To determine the location and nature of the 5' end of the *ced-3* transcript, a combination of primer extension and amplification using the polymerase chain reaction (PCR) was used. Two primers, Pex1 and Pex2, were used for primer extension. The Pex1 reaction yielded two major bands, whereas the Pex2 reaction gave one band. The Pex2 band corresponds in size to the smaller band from the Pex1 reaction, and agrees in length with a possible transcript that is trans-spliced to a *C. elegans* splice leader (Bektesh *et al., Genes & Dev.* 2:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence. The nature of the larger Pex1 band is unclear.

To confirm these observations, wild-type total RNA was reverse-transcribed and then amplified using the primers SL1 and log-5 followed by reamplification using the primers SL1 and oligo10. A product of the expected length was cloned into the PCR1000 vector (Invitrogen) and sequenced. The sequence obtained confirmed the presence of a *ced-3* message trans-spliced to

SL1 at position 2166 of the genomic sequence. These experiments suggest that a *ced-3* transcript is trans-spliced to the *C. elegans* splice leader SL1 (Bektesh *et al., Genes & Dev. 2*:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence. Based upon these observations, it is concluded that the start codon of ced-3 is the methionine encoded at position 2232 of the genomic sequence and that ced-3 is 503 amino acids in length.

The predicted ced-3 is hydrophilic (256/503 residues are charged or polar) and does not contain any obvious potential trans-membrane domains. One region of ced-3 is rich in serines: from amino acid 107 to amino acid 205, 32 of 99 amino acids are serine residues.

The sequences of 12 EMS-induced *ced-3* mutations (Table 1) were determined. Eight are missense mutations, three are nonsense mutations, and one alters a conserved G at the splice acceptor site of intron 6. Interestingly, nine of these 12 mutations alter residues within the last 100 amino acids of the protein, and none occurs within the serine-rich region.

Table 1. Sites of mutations in the ced-3 gene

Allele	Mutation	Nucleotide	Codon	Consequence
n717	G to A	6297		Altered splicing
n718	G to A	2487	65	G to R
n1040	C to T	2310	27	L to F
n1129 & n164	C to T	6434	449	A to V
n1163	C to T	7020	486	S to F
n1165	C to T	5940	403	Nonsense
n1286	G to A	6371	428	Nonsense
n1949	C to T	6222	412	Nonsense
n2426	G to A	6535	483	E to K
n2430	C to T	6485	466	A to V
n2433	G to A	5757	360	G to S

Nucleotide and codon positions correspond to the numbering in Fig. 2.

T identify functionally important regions of *ced-3*, the genomic sequences of the *ced-3* genes from the related nematode species *C. briggsae* and *C. vulgaris* were cloned and sequenced. Sequence comparison of the three *ced-3* genes showed that the relatively non-serine-rich regions of the proteins are more conserved than are serine-rich regions (Fig. 3A). All 12 EMS-induced *ced-3* mutations altered residues that are conserved among the three species. These results suggest that the non-serine-rich region is important for *ced-3* function and that the serine rich region is either unimportant or that residues within it are functionally redundant.

ced-3 protein is similar to the mammalian ICE and nedd-2 proteins

A search of the GenBank, PIR and SWISS-PROT databases revealed that the non-serine-rich regions of ced-3 are similar to hICE and mICE (Fig. 3A). The most highly conserved region among the proteins shown in Figure 3A consists of amino acids 246-360 of ced-3 and amino acids 166-287 of the hICE: 49 residues are identical (43% identity). The active site cysteine of hICE is located at cysteine 285 (Thornberry *et al., Nature 356*:768-774 (1992)). The five-amino-acid peptide (QACRG) around this active cysteine is the longest conserved peptide among mICE and hICE and ced-3.

hICE is composed of two subunits (p20 and p10) that appear to be proteolytically cleaved from a single proenzyme to the mature enzyme (Thornberry *et al., Nature 356*:768-774 (1992)). Two cleavage sites in the proenzyme, Asp-Ser at positions 103 and 297 of hICE, are conserved in ced-3 (position 131 and 371, respectively).

The C-terminal portion of ced-3 and the p10 subunit of hICE are similar to the protein product of the murine *nedd-2* gene. ced-3, nedd-2 and hICE are 27% identical (Fig. 3A). nedd-2 does not contain the QACRG peptide at the active site of hICE and mICE (Fig. 3A). Seven of eight point mutations that were analyzed (*n718, n1040, n1129, n1164, n2430, n2426 & n2433*) result in alterations of amino acids that are conserved or semi-

conserved among the three nematode ced-3 proteins, hICE and nedd-2. In particular, the mutation, n2433, introduces a Gly to Ser change near the putative active cysteine (Fig. 2, Table 1).

Discussion

The genes *ced-3* and *ced-4* are the only genes known to be required for programmed cell death to occur in *C. elegans* (Ellis *et al.*, *Cell 44*:817-829 (1986)). Genetic and molecular studies have revealed that the *ced-3* gene shares a number of features with *ced-4* (see Yuan *et al.*, *Dev. 116*:309-320 (1992)). Like *ced-4*, *ced-3* is not required for viability. It appears to contain the sequence for a single mRNA which is expressed mostly in the embryo, the stage during which most programmed cell death occurs. Furthermore, just as *ced-3* gene function is not required for *ced-4* gene expression (Yuan *et al.*, *Dev. 116*:309-320 (1992)), *ced-4* gene function is not required for *ced-3* gene expression. Thus, these two genes do not appear to control the onset of programmed cell death by acting sequentially in a regulatory transcriptional cascade. Unlike *ced-4* (Yuan *et al.*, *Dev. Biol. 138*:33-41 (1992)), *ced-3* is expressed at a substantial level in young adults. This observation suggests that *ced-3* expression is not limited to cells undergoing programmed cell death.

The ced-4 amino acid sequence is novel. Two regions show similarity to the EF-hand motif, which binds calcium (Yuan *et al.*, *Dev. 116*:309-320 (1992)). For this reason it has been suggested that ced-4 protein and hence, programmed cell death in *C. elegans*, might be regulated by calcium. ced-3 contains a region of 99 amino acids that contain 32 serines. Since serines are common phosphorylation sites (Edelman *et al.*, *Ann. Rev. Biochem. 56*:567-613 (1987)), ced-3 and hence, programmed cell death in *C. elegans*, may be regulated by phosphorylation. Phosphorylation has previously been suggested to function in cell death (McConkey *et al.*, *J. Immunol. 145*:1227-1230 (1990)). McConkey *et al.* have shown that several agents that elevate cytosolic cAMP level induce thymocyte death. This suggests that protein

kinase A may mediate cell death by phosphorylating certain proteins. Although the precise sequence of the serine-rich region varies among the three *Caenorhabditis* species studied, the relatively high number of serines is conserved in *C. elegans*, *C. briggsae* and *C. vulgaris*. None of the mutations in *ced-3* affect the serine-rich region. These observations are consistent with the hypothesis that the presence of serines is more important than the precise amino acid sequence within this region.

Much more striking than the presence of the serine-rich region in ced-3 is the similarity between the non-serine-rich regions of ced-3 and hICE and mICE.

The carboxy half of ced-3 is the region that is the most similar to ICE. A stretch of 115 residues (amino acids 246-360 of ced-3) is 43% identical between ced-3 and hICE. This region in ICE contains a conserved pentapeptide QACRG (positions 361-365 of ced-3), which surrounds the active cysteine. Specific modification of this cysteine in hICE results in complete loss of activity (Thornberry *et al., Nature 356*:768-774 (1992)). The *ced-3* mutation *n2433* alters the conserved glycine in this pentapeptide and eliminates ced-3 function. This suggests that this glycine is important for ced-3 activity and is an integral part of the active site of ICE. Interestingly, while the mutations *n718* (position 67 of ced-3) and *n1040* (position 27 of ced-3) eliminate ced-3 function *in vivo*, they contain alterations in conserved residues which are outside of the mature P20 subunit of hICE (Thornberry *et al., Nature 356*:768-774 (1992)). These residues may have a non-catalytic role in both ced-3 and ICE function, e.g., they may maintain a proper conformation for proteolytic activation. The hICE precursor (p45) is proteolytically cleaved at 4 sites (Asp103, Asp119, Asp297 and Asp316) to generate p24, p20, and p10 (Thronberry *et al., Nature 356*:768-774 (1992)). At least two of the cleavage sites are conserved in ced-3. This indicates that the ced-3 protein is processed.

The similarity between ced-3 and ICE suggests that ced-3 functions as a cysteine protease, controlling programmed cell death by proteolytically

activating or inactivating a substrate protein. A substrate for ced-3 could be the product of the *ced-4* gene which contains 6 Asp residues. These could be the target of ced-3 (Asp25, Asp151, Asp185, Asp192, Asp459 and Asp541). Alternatively, ced-3 could directly cause cell death by proteolytically cleaving certain proteins or subcellular structures that are crucial for cell viability.

ced-3 and ICE are part of a novel protein family. Thornberry *et al.* suggested that the sequence GDSPG at position 287 of hICE resembles a GX(S/C)XG motif found in serine and cysteine protease active sites (*Nature 356*:768-774 (1992)). However, in the three nematode ced-3 proteins examined, only the first glycine is conserved, and in mICE, the S/C is not present. This suggests that the *ced-3/ICE* family shares little sequence similarity with known protease families.

The similarity between ced-3 and ICE suggests not only that ced-3 functions as a cysteine protease, but also that ICE functions in programmed cell death in vertebrates. Thus, it has been observed that after murine peritoneal macrophages are stimulated with lipopolysaccharide (LPS) and induced to undergo programmed cell death by exposure to extracellular ATP, mature active IL-1β is released into the culture supernatant. In contrast, when cells are injured by scraping, IL-1β is released exclusively as the inactive proform (Hogoquist *et al., Proc. Natl. Acad. USA 88*:8485-8489 (1991)). These results suggest that *ICE* is activated upon induction of programmed cell death. *ICE* transcript has been detected in cells that do not make IL-1β (Cerretti *et al., Science 256*:97-100 (1992)), suggesting that other *ICE* substrates exist. This suggests that *ICE* could mediate programmed cell death by cleaving a substrate other than IL-1β.

The carboxy-terminal portions of both ced-3 and the p10 subunit of hICE are similar to the protein encoded by *nedd-2*. Since nedd-2 lacks the QACRG active domain, it might function to regulate ICE or ICE-like p20 subunits. Interestingly, four *ced-3* mutations alter residues conserved between nedd-2 and ced-3. Further, *nedd-2* gene expression is high during embryonic

brain development, when much programmed cell death occurs. These observations suggest that *nedd-2* functions in programmed cell death.

The *C. elegans* gene *ced-9* protects cells from undergoing programmed cell death by directly or indirectly antagonizing the activities of *ced-3* and *ced-4* (Hengartner *et al.*, *Nature 356*:494-499 (1992)). *bcl-2* also affects the onset of apoptotic cell death. Thus, if *hICE* or another *ced-3/ICE* family member is involved in vertebrate programmed cell death, *bcl-2* might act by modulating its activity. The fact that *bcl-2* is a dominant oncogene suggests that *hICE* and other *ced-3/ICE* family members might be recessive oncogenes. The elimination of such cell death genes would prevent normal cell death and promote malignancy, just as overexpression of *bcl-2* does.

Example 2

The mouse *ICE* homolog (*mICE*) from a mouse thymus cDNA library (Stratagene) was cloned by low stringency hybridization using *hICE* as a probe. The clone is identical to the clone isolated by Nett *et al.* (*J. Immun. 149*:3245-3259 (1992)) except that base pair 166 is an A and encodes Asn rather than Asp. This may be a DNA polymorphism because the clone was derived from a B6/CBAFlJ (C57Black x CBA) strain cDNA library (Stratagene), while the Nett *et al.* clone was derived from a WEH13 cDNA library (Stratagene). Subsequent experiments have shown that this variation is not in a region essential for *ICE* function (see below).

A transient expression system was developed to determine if overexpression of *mICE* kills cells. *mICE* cDNA was fused with the *E. coli lac-Z* gene and placed under the control of the chicken β-actin promoter (Fig. 4). To test the function of the subunits, P20 and P10, which are processed from a precursor peptide, two additional fusion genes were made (*P20/P10-lacZ* and *P10-lacZ*).

The constructs, shown in Fig. 4, were transfected into Rat-1 cells by calcium phosphate precipitation. 24 hours after transfection, cells were fixed

and X-gal was added. Healthy living rat cells are flat and well-attached to plates, while dying cells are round and often float into the medium. After 3 hours of color development, most blue cells transfected with intact *mICE-lacZ* or *P20/P10-lacZ* were round. However, most blue cells transfected with *P10-lacZ* or the control *lac-Z* construct were normal flat cells (Table 2). Similar results were obtained with NG108-15 neuronal cells (not shown).

Table 2. Overexpression of *mICE* causes Rat-1 cells to undergo programmed cell death

The constructs shown in Fig. 4 were transiently transfected into Rat-1 cells, Rat-1 cells expressing *bcl-2* (Rat-1/*bcl-2*) or Rat-1 cells expressing *crmA* (Rat-1/*crmA*). 24 hrs after transfection, cells were fixed and stained with X-gal for 3 hrs. The data shown are the percentage of round blue cells among total number of blue cells. The data were collected from at least three different experiments.

Construct	Rat-1	Rat-1/*bcl-2*	Rat-1/*crmA*
pactβgal'	1.44 ± 0.18	2.22 ± 0.53	2.89 ± 0.79
pβactM10Z	80.81 ± 2.33	9.91 ± 2.08	18.83 ± 2.86
pβactM11Z	93.33 ± 2.68	13.83 ± 4.23	24.48 ± 2.78
pβactM19Z	2.18 ± 0.54	-	-
pβactM12Z	2.44 ± 0.98	3.33 ± 1.45	2.55 ± 0.32
pβact17Z	2.70 ± 1.07	-	-
pJ485	1.32 ± 0.78	-	-
pβactced38Z	46.73 ± 4.65	35.28 ± 1.36	34.40 ± 2.38
pβactced37Z	3.67 ± 1.39	-	-

Methods: a: Construction of *bcl-2* expression vector (pJ415): pJ415 was constructed by first inserting 5', the 400bp *Bgl*II/*Bam*HI *crmA* fragment into the *Bam*HI site of the pBabe/puro vector and then inserting the remaining 1kb *Bam*HI *crmA* fragment into the 3'*Bam*HI site in the sense direction. b: Construction of the *bcl-2* expression vector (pJ436): pJ436 was constructed by inserting an *Eco*RI/*Sal*I *bcl-2* fragment into the *Eco*RI/*Sal*I sites of the pBabe/puro vector. c: Establishing Rat-1 cell lines that overexpress *crmA* and *bcl-2*: pJ415 and pJ436 were electroporated into ΨCRE retroviral packaging cells (Danos *et al., Proc. Natl. Acad. Sci. (USA) 85*:6460-6464 (1988))

using a BioRad electroporating apparatus. Supernatant either from overnight transiently transfected ΨCRE cells or from stable lines of ΨCRE cells expressing either *crmA* or *bcl-2* were used to infect Rat-1 cells overnight in the presence of 8 μg/ml of polybrene. Resistant cells were selected using 30 ug/ml puromycin for about 10 days. Resistant colonies were cloned and checked for expression using both Northern and Western blots. Bcl-2 antibodies were from S.J. Korsmeyer and from DAKO. crmA antiserum was made by immunizing rabbits with an *E. coli*-expressed crmA fusion protein (pJ434). pJ434 was made by inserting an *Eco*RI/*Sal*I fragment of *crmA* cDNA into *Eco*RI/*Sal*I sites of pET21a (Novagen) and fusion protein was expressed in the *E. coli* BL21 (DE3) strain. Multiple lines that express either *bcl-2* or *crmA* were checked for suppression of *mICE* induced cell death and all showed similar results.

When cells were stained with rhodamine-coupled anti-β galactosidase antibody and Hoechst dye, it was found that β-galactosidase-positive round cells had condensed, fragmented nuclei. Such nuclei are indicative of programmed cell death. When observed with an electron microscope, the X-gal reaction product was electron dense, allowing cells expressing *mICE-lacZ* to be distinguished from other cells (Snyder *et al.*, *Cell 68*:33-51 (1992)). The cells expressing the chimeric gene showed condensed chromatin and membrane blebbing. These are characteristics of cells undergoing programmed cell death (Wyllie, A.H., in *Cell Death in Biology and Pathology*, 9-34 (1981); Oberhammer *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 89*:5408-5412 (1992); Jacobson *et al.*, *Nature 361*:365-369 (1993)). Thus, the results indicate that overexpression of *mICE* induces programmed cell death and that induction depends on both P20 and P10 subunits.

When color development in Rat-1 cells transfected with *mICE-lacZ* or *P20/P10-lacZ* is allowed to proceed for 24 hours, a greater number of flat cells become blue. This result indicates that cells tolerate lower levels of *ICE* activity.

If *mICE* is a vertebrate homolog of *ced-3*, then *ced-3* might also be expected to cause cell death in vertebrates. This hypothesis was tested by making a *ced-3-lacZ* fusion construct and examining its ability to cause cell

death using the assay as described above. As expected, the expression of *ced-3* caused the death of Rat-1 cells (Table 2).

If *mICE* functions in a similar way to *ced-3*, mutations eliminating *ced-3* activity in *C. elegans* should also eliminate its activity in vertebrates. This hypothesis was tested by mutating the Gly residue in the pentapeptide active domain of hICE, QACRG, to Ser. It was found that this mutation eliminated the ability of both *mICE* and *ced-3* to cause rat cell death in Rat-1 cells (Table 2).

crmA specifically inhibits *ICE* activity (Ray *et al.*, above). To demonstrate that cell death associated with overexpression of *mICE* is due to the enzymatic activity of mICE, Rat-1 cells were infected with a pBabe retroviral construct (Morgenstern *et al.*, *Nucl. Acids Res. 18*:3587-3596 (1990)) expressing *crmA* and cell lines identified which produce high levels of *crmA*. When the *mICE-lacZ* construct was transfected into these cell lines, it was found that a large percentage of blue cells had a healthy, flat morphology (Table 2). In addition, a point mutation that changes the Cys residue in the active site pentapeptide, QACRG to a Gly eliminates the ability of *mICE* to cause cell death (construct pβactM17Z, Figure 4, Table 2). This result indicates that the proteolytic activity of *mICE* is essential to its ability to kill cells.

bcl-2 can also prevent or inhibit cell death (Vaux *et al.*, Nuñez *et al.*, Strasser *et al.*, Sentman *et al.*, above). Rat-1 cells were infected with the pBabe retroviral construct expressing *bcl-2*. Transfection of the *mICE-lacZ* fusion construct into the cells lines overexpressing *bcl-2* showed that a high percentage of blue cells were now healthy (Table 2). Thus, cell death induced by overexpression of *mICE* can be suppressed by *bcl-2*. This result indicates that cell death induced by overexpression of *mICE* is probably caused by activation of a normal programmed cell death mechanism. Thus, taken together, the results all suggest that vertebrate animals have a genetic pathway of programmed cell death similar to that of *C. elegans* (Fig. 5).

Example 3

As described above, the genes in the *ICE/ced-3* family would be expected to function during the initiation of programmed cell death. In order to identify additional members of this gene family, cDNA encoding hICE was used to screen a mouse thymus cDNA library (Stratagene) under conditions of low stringency. Using this procedure, a new gene was identified and named "*mIch-2*" (see Figure 6 for the cDNA sequence and deduced amino acid sequence of *mIch-2*).

Figures 7 and 7A show that the protein encoded by *mIch-2* contains significant homology to hICE, mICE, and *ced-3*. The sequence homology indicates that *mIch-2*, like *mICE*, is a vertebrate cell death gene.

Northern blot analyses showed that the expression of *mIch-2*, unlike *mICE*, which is broadly expressed during embryonic development, is restricted to the thymus and placenta, areas in which cell death frequently occurs. In addition, it was found that the expression of *mIch-2* in the thymus can be induced by dexamethasone, an agent which causes thymus regression. It is concluded that *mIch-2* is a thymus/placenta specific vertebrate cell death gene.

Example 4

Extensive cell death occurs in the developing nervous system (Oppenheim, R. W., *Ann. Rev. Neurosci. 145*:453-501 (1991)). Many neurons die during the period of synapse formation. During this critical period, the survival of neurons depends on the availability of neural trophic factors. The survival of isolated primary neurons *in vitro* depends critically on the presence of such trophic factors (Davies, A. M., *Development 100*:185-208 (1987)). Removal of such factors induces neuronal cell death, usually within 48 hrs. The death of the sympathetic neurons and sensory neurons whose survival depends on one or more members of the nerve growth factor

family (nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3) can be prevented by microinjection of a *bcl-2* expression vector (Garcia, I., *et al.*, *Science 258*:302-304 (1993); Allsopp *et al.*, 1993). To examine if the genes in the *ICE/ced-3* family are involved in neuronal cell death, the ability of *crmA* (which inhibits *ICE*) to inhibit the death of chicken dorsal root ganglionic neurons induced by NGF removal was examined. It was found that microinjection of an expression vector containing *crmA* inhibits the death of DRG neurons as effectively as that of a *bcl-2* expression vector (Gagliardini, V., *et al.*, *Science 263*:826-828 (1994)). This result demonstrates that the genes in the *ICE/ced-3* family play a role in regulating neuronal cell death during development.

Example 5

Results

Cloning of Ich-1

The protein product of the *C. elegans* cell death gene, *ced-3*, is homologous to the product of the mouse gene, *nedd*-2. The *nedd*-2 cDNA in the data bank has an open reading frame of 171 amino acids and has long 3′ and 5′ untranslated regions. This 171-amino acid nedd-2 protein does not contain the active domain, QACRG, of ICE and ced-3 proteins and is homologous only to the P10 subunit of mammalian ICE and the C-terminal part of ced-3. While analyzing *nedd*-2 cDNA, the inventors discovered that it contains a sequence that can potentially encode a QACRG pentapeptide, but that the sequence is in another reading frame. The inventors considered the possibility that the *nedd-2* cDNA isolated by Kumar *et al.* contains cloning artifacts and that another *nedd-2* transcript encodes a protein homologous to both the P20 and P10 subunits of ICE.

A mouse *nedd-2* probe was made by polymerase chain reaction (PCR). Using this probe, three cDNA libraries were screened: a mouse embryonic day 11.5 cDNA library from CLONTECH (one million clones screened), a human fetal brain cDNA library from James Gusella's laboratory (10 million clones screened), and a human fetal brain cDNA library from Stratagene (one million clones screened). The longest positive cDNA clones were obtained from the Stratagene cDNA library. From the Stratagene library, two cDNA species (pBSH37 and pBSH30) were identified that encode two closely related proteins homologous to the mouse nedd-2 (Figures 10-12).

The insert of pBSH37 (2.5 kb) encodes a protein of 435 amino acids that contains amino acid sequence similarities to both the P20 and P10 subunits of hICE and the entire ced-3. The insert of pBSH30 (2.2 kb) has an open reading frame of 512 amino acids and contains an additional 61 bp one basepair after the sequence encoding QACRG. This causes an early termination of protein translation. The Northern blot analysis showed that expression of this human gene is different than expression of *nedd-2* (Kumar *et al.*); thus, the sequences were renamed *Ich-1*$_L$ (pBSH37) (Figure 10A) and *Ich-1*$_S$ (pBSH30) (Figure 10B).

A comparison of cDNA sequences revealed that *Ich-1*$_S$ cDNA differs from *Ich-1*$_L$ at the 5′ end, around the beginning of the initiation of translation, and by the presence of an additional intron in the middle of *Ich-1*$_S$ cDNA (Figure 11B). The first difference is at the beginning of the coding region. The putative first methionine of *Ich-1*$_S$ is 15 amino acids downstream from the first methionine of *Ich-1*$_L$; the first 35 bp of *Ich-1*$_S$ are different from *Ich-1*$_L$ and include a stop codon (Figures 10A and 10B). PCR analysis using primers specific to the first 35 bp of *Ich-1* and the *Ich-1*$_S$-specific intron (see below), and human placenta cDNA as template, amplified a DNA fragment of predicted size. This suggests that the 35 bp *Ich-1*$_S$-specific sequence is not a cloning artifact and is present in the endogenous *Ich-1*$_S$ mRNA.

The second difference is distal to the active domain QACRG. *Ich-1*$_S$ begins to differ from *Ich-1*$_L$ one basepair after the coding region of the active

site QACRG. The difference is caused by a 61 bp insertion, which results in a termination codon 21 amino acids downstream from the insertion. The last two identical basepairs of *Ich-1*$_S$ and *Ich-1*$_L$ are AG, the general eukaryotic splicing donor consensus sequence (Mount, 1982).

To eliminate the possibility that the 61 bp insertion in pBSH30 is a result of incomplete RNA processing, both forms of murine *Ich-1* were cloned from adult mouse brain mRNA by PCR using primers flanking the insertion site as described (Experimental Procedure). The resulting 233 and 172 bp fragments (Figure 11B) were cloned separately and sequenced. Three murine *Ich-1*$_L$ clones and two murine *Ich-1*$_S$ clones were sequenced. Sequencing confirmed that the murine *Ich-1*$_S$ contains the same 61 bp insertion as in human *Ich-1*$_S$ at the same position (Figure 11A).

Chicken *Ich-1* from an embryonic chicken cDNA library (Clontech) was also cloned using a chicken *Ich-1* probe obtained by PCR (see Experimental Procedures). Two clones were isolated. One encodes *Ich-1*$_L$ and the other encodes *Ich-1*$_S$ which contains a 62 bp insertion at the same position. The DNA sequence of the 62 bp insertion is 72% identical to that of human and murine *Ich-1*$_S$ and also caused premature termination of protein translation. The extra basepair in the intron of chicken *Ich-1*$_S$ causes the amino acid sequence of the last 41 amino acids of chicken *Ich-1*$_S$ to differ from human and murine *Ich-1*$_S$; however, truncation of the protein may be the important point.

To examine the origin of the 61 bp insertion in murine and human *Ich-1*$_S$, mouse genomic *Ich-1* DNA was cloned. Analysis showed that the 61 bp is from an intron whose sequence is identical in human and mouse *Ich-1*. The difference between *Ich-1*$_S$ and *Ich-1*$_L$ is caused by alternative splicing from the two different 5′ splicing donor sequences. The first two basepairs of the 61 bp intron and the two basepairs after the 61 bp intron are GT (Figure 11A). This sequence is the 100% conserved general eukaryotic splicing donor consensus sequence (Mount, 1982). The DNA sequence at the 3′ splicing acceptor site is AG. This sequence is the 100% conserved

eukaryotic splicing acceptor sequence (Figure 11A). Thus, the DNA sequences at the splicing junction are completely consistent with alternative splicing of *Ich-1$_S$*.

As the result of an insertion of an intron between coding regions, the open reading frame of *Ich-1$_S$* is divided into two: the first encodes a 312 amino acid peptide homologous to the P20 subunit of hICE. The second encodes a 235 amino acid peptide homologous to a part of the P20 subunit and the P10 subunit of hICE. The second is nearly identical to mouse *nedd-2* (Figure 10B). The data suggest that only the first open reading frame is translated in cells. A schematic diagram of *Ich-1$_L$* and *Ich-1$_S$* is shown in Figure 11B.

Ich-1$_L$ contains similarities to both ICE (27% identity and 52% similarity) and ced-3 (28% identity and 52% similarity) (Figures 12A and 12B). Thus, the homology between Ich-1 and ced-3, Ich-1 and ICE is about equal.

Ich-1 is expressed in many tissues and THP-1 cells which express interleukin-1β converting enzyme

To characterize the function of *Ich-1*, the expression pattern of *Ich-1* was examined. Northern blot analysis of human fetal heart, brain, lung, liver and kidney tissue was done using the insert of pBSH37 as a probe. The probe hybridizes to both *Ich-1$_S$* and *Ich-1$_L$* transcripts. The analysis showed that 4 kb *Ich-1* mRNA is expressed at the same low levels in all tissues examined. When the same Northern blot (completely stripped of the previous probe) was analyzed using the *Ich-1$_S$* 61 bp intron as a probe (which hybridizes to *Ich-1$_S$* transcript only), it showed that *Ich-1$_S$* is expressed in a larger amount in the embryonic heart and brain than in the lung, liver, and kidney. This result demonstrates that in the embryonic lung, liver and kidney, *Ich-1$_L$* is expressed to a greater extent than *Ich-1$_S$* is. In Northern blot analysis of adult RNA with the pBSH37 probe, *Ich-1* is detected in all the tissues examined. The level is

higher in placenta, lung, kidney and pancreas than in heart, brain, liver and skeletal muscle.

To study the expression of *Ich-1*$_L$ and *Ich-1*$_S$ during mouse embryonic development, a quantitative RT-PCR analysis was developed using specific primers that differentiate between *Ich-1*$_L$ and *Ich-1*$_S$. Primers were synthesized that flank the 61 bp intron sequence of *Ich-1*$_S$. The two primers are located in separate exons separated by a 2.8 kb intron in genomic DNA. Thus, the possibility of genomic DNA contamination was eliminated. *Ich-1*$_L$ and *Ich-1*$_S$ were amplified simultaneously to produce DNA fragments of 172 bp and 233 bp, respectively. The cDNA templates were reverse-transcribed from mRNA isolated from thymus, adult heart, adult kidney, embryonic 15d brain, and adult brain. Negative (no DNA template) and positive (*Ich-1*$_S$ and *Ich-1*$_L$) controls were used. Actin primers were used on one set of each sample. Analyses showed that only expression of *Ich-1*$_L$ can be detected in thymus while the expression of both *Ich-1*$_L$ and *Ich-1*$_S$ can be detected in heart, kidney, and both embryonic and adult brain. The expression of *Ich-1*$_S$ was found to be highest in embryonic brain by this PCR analysis. The results are consistent with Northern blot analysis described above. The results were reproducible among multiple mRNA preparations.

To examine whether *Ich-1* and *hICE* are expressed in the same cells, a Northern blot of THP-1 and U937 cells was analyzed with the *Ich-1* probe, pBSH37. *hICE* expression has been detected in these cells (Thornberry, N. A., *et al.*, *Nature 356*:768-774 (1992); Cerretti, D. P., *et al.*, *Science 256*:97-100 (1992)). The inventors found that *Ich-1* can be detected in THP.1 and U937 cells. Thus, both *Ich-1* and *hICE* are expressed in THP.1 and U937 cells.

Ich-1$_S$, *Ich-1*$_L$ and *hICE* expression were compared in human cell lines. Using a similar quantitative RT-PCR analysis, the expression of *Ich-1*$_L$, *Ich-1*$_S$ and *hICE* were compared in HeLa, Jurkat, THP.1 and U937 cells. *Ich-1*$_L$ and *Ich-1*$_S$ were amplified simultaneously to produce DNA fragments of 234 bp and 295 bp, respectively. *hICE* was amplified as a fragment of 191 bp.

cDNA templates were reverse-transcribed from mRNA isolated from HeLa, Jurkat, THP.1, and U937 cells. Negative (no DNA template) and positive (*hICE* cDNA) controls were used. pBSH37 and pBSH30 were used as positive controls for *Ich-1*$_L$ and *Ich-1*$_S$ expression. Chicken actin cDNA was used as a positive control for actin. Expression of *Ich-1*$_S$ was detected in HeLa and Jurkat cells but not in THP.1 and U937 cells. Both *hICE* and *Ich-1* transcripts are present in relatively high levels in HeLa cells. The level of *Ich-1* transcript is higher than that of *hICE* transcript in Jurkat cells. Both *hICE* and *Ich-1* expression is detected in THP.1 and U937 cells.

Using a quantitative RT-PCR analysis, the inventors examined the expression of *hICE* and *Ich-1* in the normal living T-cell hybridoma DO11.10 cells (Haskins, K., *et al.*, *Exp. Med. 157*:1149-1169 (1983)) and dying DO11.10 cells (serum-deprived). The expression of both *hICE* and *Ich-1* can be detected in DO11.10 cells. Interestingly, the expression levels of both *Ich-1*$_L$ and *hICE* appear to increase in dying DO11.10 cells.

Overexpression of Ich-1*$_L$ *induces rat-1 fibroblast death

To examine the function of *Ich-1*$_L$, the same transient expression system used for *ICE* (Miura, M., *et al.*, *Cell 75*:653-660 (1993)) was used to determine if overexpression of *Ich-1* induces programmed cell death. The human *Ich-1*$_L$ cDNA was fused with the *E. coli lacZ* gene and the fused gene was placed under the control of the chicken β-actin promotor (pβactH37Z). This fusion gene was transfected into Rat-1 cells by lipofectamine-mediated gene transfer and the expression of the gene was examined using the X-gal reaction. Results showed that most of the blue (X-gal-positive) Rat-1 cells transfected with pβactH37Z were round. These results are similar to those obtained with cells transfected with *mICE-lacZ* fusion sequence shown in Table 2. In contrast, most blue cells transfected with vector alone were flat and healthy. This result suggests that the expression of *Ich-1*$_L$ induces Rat-1 cells to die.

To examine whether the cell death induced by *Ich-1* has any cell type specificity and to compare its effect with that of *mICE*, *mICE-lacZ* and *Ich-1-lacZ* fusion constructs were transfected to HeLa cells, NG108-15 cells, Rat-1 cells, and COS cells. These cells thus expressed *mICE-lacZ* (pβactM10Z) *Ich-1$_L$-LacZ* (pβactH37Z) and *Ich-1$_S$-lac-Z* (pβactH30Z1) and *lacZ* control (pactβgal'). The cell killing effect was assayed as for Table 2. The results are shown in Table 3.

TABLE 3

EFFECTS OF ICH-1 OVEREXPRESSION ON CELLS IN CULTURE

Expression cassettes	COS	HeLa	NG108-15	Rat-1	Rat-1/bcl-2	Rat-1/*crmA*
pactβgal'	• 1.3+0.1(983)	2.9±0.2(1020)	4.2±0.2(1535)	2.9±0.2(1470)	3.4±0.2(1446)	3.7±0.1(1459)
pβactM10Z	• 11.0±0.2(1080)	93.9±0.3(1003)	80.2±0.5(1545)	94.2±1.1(978)	28.8±0.5(691)	45.8±1.6(233)
pβactH37Z	8.3±0.9(1053)	91.4±0.2(1076)	68.7±1.5(1605)	92.1±0.3(1079)	21.5±3.2(1335)	80.7±0.9(1010)
pβactH37ZCS	ND	5.6±0.1(1039)	5.9±0.9(707)	4.1±0.2(1477)	ND	ND
pβactH37ZAT	ND	8.2±0.7(435)	5.2±0.2(640)	5.4±0.3(1356)	ND	ND
pβactH30Z1	1.3±0.2(676)	0.0±0.0(40)	0.0±0.0(61)	1.8±0.4(785)	ND	ND

The *mICE-LacZ* (pβactM10Z), *Ich-1$_L$-lacZ* (pβactH37Z), *Ich-1$_L$*(S303C)-*lacZ* (pβactH37ZCS), *Ich-1$_L$*(T352A)-*lacZ* (pβactH37ZAT), *Ich-1$_S$-lacZ* (pβactH30Z1) and control vector alone (pactβgal') were transiently transfected into Rat-1 cells, Rat-1 cells expressing human *bcl-2*, Rat-1 cells expressing cowpox virus *crmA* gene, HeLa cells, NG108-15 cells and COS cells. Cells were fixed lightly 24 hr after transfection and stained with X-Gal for 3 hr. The data (mean±SEM) shown are the percentage of round blue cells among total number of blue cells counted. The numbers in the parentheses are the number of blue cells counted. The data were collected from at least three independent experiments. ND = not determined.

Compared to controls, the cytotoxic effect of *Ich-1* and *mICE* exhibit certain cell type specificities. Expression of *Ich-1* or *mICE* kills Rat-1 cells and HeLa cells effectively (>90% dead). NG108 cells are more resistant to *Ich-1* and *mICE* expression than Rat-1 cells and HeLa cells (68-80% dead). Expression of *Ich-1* or *mICE* cannot kill COS cells.

To confirm that the cell death caused by *Ich-1*$_L$ expression is apoptosis, the inventors examined the nuclear morphology of the cell death induced by *Ich-1* expression. Rat-1 cells were transiently transfected with control pactβgal' vector and 24 hours later, fixed and stained by anti-β-galactosidase antibody or by Hoechst dye 33258 using a protocol from Miura *et al.* (1993). The nuclear morphology in β-galactosidase-expressing cells is normal and non-condensed. Rat-1 cells were also transiently transfected with pβactH37Z expressing *Ich-1*$_L$. The nuclei of round cells expressing the *Ich-1*$_L$*-lacZ* chimeric gene were condensed and fragmented. This is one of the characteristics of cells undergoing apoptosis. Thus, the results suggest that overexpression of *Ich-1*$_L$, like that of *mICE*, causes Rat-1 cells to undergo programmed cell death.

To determine the structure and function of *Ich-1*$_L$ protein, two mutant *Ich-1*$_L$ fusion proteins were made: the first is a Ser → Cys 303 in the active site of Ich-1$_L$; the second is a Thr → Ala 352 in the putative P10 subunit (Fig. 12A). The Ala 352 in P10 is an amino acid residue of ced-3 that is conserved in Ich-1$_L$ but not in ICE. The mutant *Ich-1*$_L$*-lacZ* fusion constructs were transfected into Rat-1 cells and expression was examined by the X-gal reaction.

The analysis revealed that the S303C (pβactH37ZCS) and T352A (pβactH37ZAT) mutations eliminated the activity of Ich-1$_L$ completely (Table 3). These results suggest that the ability of Ich-1$_L$ to cause cell death depends upon its enzymatic activity and that only some characteristics of ced-3 are conserved in Ich-1$_L$.

The cell death induced by overexpression of *ICE* can be inhibited by *bcl-2* and *crmA* (Miura, M., *et al.*, *Cell* 75:653-660 (1993)). To examine if

the cell death induced by expression of *Ich-1*$_L$ could also be inhibited by *bcl-2* and *crmA*, *Ich-1*$_L$-*lacZ* fusion construct (pβactH37Z) was transfected into Rat-1 cells that overexpress either *bcl-2* or *crmA*. Cell death was assayed as described for Table 3. The results showed that the cell death induced by overexpression of *Ich-1*$_L$ could be inhibited effectively by *bcl-2* but only marginally by *crmA*.

Expression of Ich-1*$_S$ *protects Rat-1 fibroblast death induced by serum removal

Since *Ich-1*$_S$ contains two open reading frames, it was important to determine which reading frame is functionally translated (Figure 11A). *Ich-1*$_S$ was translated in the presence of ^{35}S-methionine using *in vitro* transcribed RNA in a reticulocyte lysate as described in Experimental Procedures. The translated products were run on an SDS-polyacrylamide gel with molecular weight standards. *Ich-1*$_S$ antisense RNA was used as a negative control. Results showed that only the first reading frame was translated.

Second, *E. coli lacZ* gene was fused to the ends of the first (pβactH30Z1) and second (pβactH30Z2) open reading frames. The constructs were separately transfected into Rat-1 cells and the cells were assayed for color using the X-gal reaction. Results showed that when the *lacZ* gene was fused to the end of the first open reading frame, blue cells could be detected. Blue cells were not detected when the *lacZ* gene was fused to the second open reading frame. Thus, it is likely that only the first open reading frame is used *in vivo*.

To characterize the function of *Ich-1*$_S$, the ability of pβactH30Z1 to cause cell death was examined. pβactH30Z1 was transfected into Rat-1 cells, COS cells, HeLa cells and NG108-15 cells, and the X-gal reaction was developed as before. The analysis showed that the expression of pβactH30Z1 did not cause cell death (Table 3).

To examine if *Ich-1$_S$* has any protective effect against cell death, a stable Rat-1 cell line that expresses *Ich-1$_S$* was established. The cDNA *Ich-1$_S$* was cloned into pBabepuro retroviral expression vector (Morgenstern *et al.*, *Nucl. Acids Res. 18*:3587-3596 (1990)) and transfected into Rat-1 cells. The stable transfectants were selected in puromycin and individual clones were assayed for expression of *Ich-1$_S$* by Northern blot analysis. The clones that expressed *Ich-1$_S$* were used for analysis and the clones that did not express *Ich-1$_S$* were used as negative controls together with untransfected Rat-1 cells. Nomarski micrographs were taken on days 0, 2, 3, and 4 of control Rat-1 cells, *Ich-1$_S$* non-expressing Rat-1 cells, *crmA* expressing Rat-1 cells, and *bcl-2* expressing Rat-1 cells in serum-free medium. Trypan blue assay was also performed.

When plated to non-confluent density and washed carefully, 90% of Rat-1 cells die in serum-free medium. However, under these conditions, Rat-1 cells expressing *bcl-2* or *crmA* are resistant to death (Fig. 13). When the ability of the stable Rat-1 cell lines that express human *Ich-1$_S$* was tested under serum-free conditions, it was found that they are more resistant to serum deprivation than parental Rat-1 cells and negative control transfectants not expressing *Ich-1$_S$* (Fig. 13). These experiments suggest that *Ich-1$_S$* has the ability to prevent cell death.

Ich-1$_S$ may prevent cell death by inhibiting *Ich-1$_L$*. The inventors thus examined whether Rat-1 cells express *Ich-1*. Using mouse *Ich-1* cDNA as a probe, an mRNA species predictive of the *Ich-1* transcript was detected in Rat-1 cells under low stringency conditions.

Discussion

The isolation and characterization of *Ich-1*, a mammalian gene belonging to the cell death gene family of *ICE/ced-3*, has been described. Two distinct *Ich-1* mRNA species have been identified (*Ich-1$_L$* and *Ich-1$_S$*). These two cDNAs differ in the 5' region around the translation initiation and

in the middle region. The difference in the middle is the result of alternative use of two different 5′ splicing donor sites.

The *Ich-1* gene is expressed at low levels in embryonic and adult tissues. *Ich-1*$_S$ is expressed at higher levels than *Ich-1*$_L$ in embryonic heart and brain. The converse is true in embryonic lung, liver and kidney. Expression of Ich-1$_S$ can be detected in all tissues examined except thymus. The expression of both *hICE* and *Ich-1* can be detected in THP.1 cells, HeLa cells, Jurkat cells, U937 cells, and DO11.10 cells. The expression of both *hICE* and *Ich-1*$_L$ appears to increase in dying cells under serum deprived conditions. Overexpression of *Ich-1*$_L$ in rat fibroblast cells caused programmed cell death prevented by *bcl-2*. This suggests that *Ich-1* is a programmed cell death gene. Overexpression of *Ich-1*$_S$, however, did not cause cell death. Stable expression of *Ich-1*$_S$ prevented Rat-1 cell death induced by serum deprivation. The collective results show that *Ich-1* encodes protein products that regulate cell death positively and negatively.

The mouse *nedd*-2 gene was originally isolated by Kumar *et al.* (*Biochem. & Biophy. Res. Comm. 185*:1155-1161 (1992)). The *nedd*-2 gene was identified as having a transcript of 3.7 kb that is abundantly expressed in embryonic day 10 mouse brain and almost undetectable in adult brain. The *nedd*-2 cDNA isolated contained an open reading frame of 171 amino acids and long 5′ and 3′ untranslated regions with stop codons in all reading frames. The 171-amino-acid open reading frame is homologous to P10 subunit of hICE and the C-terminal part of ced-3 (Yuan, J., *et al.*, *Cell 75*:641-752 (1993)). The amino acid sequence of the C-terminal part of Ich-1$_L$ is 87% identical to the amino acid sequence of the mouse nedd-2 protein from residues 42 to 172 (the first 41 amino acids are different because of the presence of the 61 bp intron). In mouse, *nedd-2* is a unique gene (L. Wang, unpublished data). Thus, human *Ich-1* and mouse *nedd-2* must be the same gene.

In the Northern blot analysis described herein, *Ich-1* expression in human fetal brain is not high compared to other tissues tested (heart, lung, liver and kidney) and does not appear to be significantly down-regulated in

adult brain. Part of the difference could be explained by the different developmental stages tested: mouse E10 versus human 20-26 week old fetuses. However, *Ich-1* expression can be detected in human and mouse adult tissues.

In the studies herein, amplification of the 5′ untranslated regions of the mouse *nedd*-2 cDNA that Kumar *et al.* reported was not achieved. It is possible that the 5′ untranslated region in the Kumar *et al.* clone was a product of incompletely processed *nedd*-2 mRNA. Both *Ich-1* mRNAs are about 4 kb; since the cDNA clones described herein are 2.5 kb and 2.2 kb for *Ich-1*$_L$ and *Ich-1*$_S$, respectively, these cDNAs are incomplete. However, since they are fully functional in the assay reported herein, the complete coding regions should be encoded in these two cDNAs.

Ich-1 is a new member of the *ICE/ced-3* family of cell death genes. Thus, unlike *C. elegans*, mammals must have multiple members of *ICE/ced-3*. *Ich-1* is even slightly more homologous to *ced-3* than *mICE*. The cell death induced by overexpression of *Ich-1* was poorly inhibited by *crmA*. This result is similar to that with *ced-3* (Miura, M., *et al.*, *Cell* 75:653-660 (1993)).

The nucleotides corresponding to the two amino acid residues of ced-3 that are conserved in Ich-1, but not in ICE, were mutagenized. Results showed that T352A completely eliminated the ability of Ich-1 to cause cell death, despite the fact that the corresponding amino acid in ICE is a Ser. These data also suggest that *Ich-1* is mechanistically more similar to *ced-3* than *ICE*, and that *Ich-1* and *ICE* may have evolved independently from *ced-3*.

The overexpression of *ICE* and *Ich-1* can kill Rat-1 cells and HeLa cells effectively but NG108 cells only moderately. It is possible that NG108 cells express a higher level of *ICE* and *Ich-1* inhibitors. COS cells are completely resistant to the cell killing activity of *ICE* and *Ich-1*. COS cells may lack either the activator or the substrates of ICE and Ich-1. This result also suggests that the cytotoxic effects of ICE and Ich-1 have certain specificity and are unlikely to be caused by random cleavage activities of proteases.

Ich-1 encodes protein products that prevent or cause cell death, depending on how the mRNA is processed. Similar regulation has been observed with *bcl-x*, a *bcl-2* related gene (Boise *et al.*, 1993). The *bcl-x* transcripts can also be processed in two different ways: the larger mRNA, *bcl-x*$_L$, encodes a *bcl-2* related protein product that can inhibit cell death. Alternative splicing of *bcl-x* transcript generates another smaller transcript, *bcl-x*$_S$. This encodes an internal truncated version of bcl-x that inhibits the ability of *bcl-2* to enhance the survival of growth factor-deprived cells. Control of the RNA splicing may be an important regulatory point in programmed cell death.

Ich-1$_S$ could act to prevent cell death by inactivating the activator of cell death or by directly inactivating *Ich-1*$_L$. In the transient transfection assay, the expression of *Ich-1*$_L$-lacZ fusion gene and the *ICE*-lacZ fusion gene kill the stable *Ich-1*$_S$-expressing cells as efficiently as the control Rat-1 cells (L. Wang, unpublished data). Thus, unlike *crmA* or *bcl-2*, the inhibition of cell death by *Ich-1*$_S$ may be highly dosage-dependent. This could explain why the expression of *Ich-1*$_S$ provided only partial protection of serum deprived Rat-1 cells. Possibly, only cells expressing high levels of *Ich-1*$_S$ are protected.

crmA has the ability to suppress cell death induced by overexpression of *Ich-1*$_L$. The amino acid sequence of crmA is homologous to the members of the serpin superfamily (Pickup *et al.*, 1986), which usually inhibit serine proteases by acting as pseudosubstrates. The nature of interaction of ICE and crmA protein is likely to be similar to the interaction of other serpin and serine proteases. The inhibition of *ICE* family members by *crmA* may depend upon both the affinity and relative concentration of ICEs and crmA. The fact that *crmA* can suppress a certain percentage of cell deaths induced by overexpression of the *Ich-1*$_L$ suggests that crmA and Ich-1 can bind to each other. It is possible that when the Ich-1 concentration is lower, crmA may be able to suppress cell death induced by Ich-1 to a greater extent. Microinjection of *crmA* expression construct can effectively suppress the death of dorsal root ganglia neurons induced by nerve growth factor deprivation

(Gagliardini, V., *et al.*, *Science 263*:826-828 (1994)). One or more *ICE/ced-3* family members may be responsible for neuronal cell death. When *crmA* expression construct is microinjected into neurons, the transient concentration of crmA may be very high. Thus, it is possible that *crmA* may be able to suppress multiple members of *ICE/ced-3* family under such conditions despite the fact that their affinity to crmA is not very high.

Since the expression of *Ich-1* and *ICE* can be detected in the same cells, the results described herein suggest that multiple members of *ICE/ced-3* family may contribute to cell death induced by a single signal. There are three possible ways that *Ice* and *Ich-1* may act to cause cell death. First, *Ich-1* may activate *ICE*, directly or indirectly, to cause cell death. Second, *ICE* may inactivate *Ich-1*, directly or indirectly, to cause cell death. Third, *ICE* and *Ich-1* may act in parallel to cause cell death. In the first scenario, the inhibitor of *ICE* should inhibit cell death induced by *Ich-1*. In the second scenario, the inhibitor of *Ich-1* should inhibit the cell death induced by *ICE*. To test this hypothesis, specific inhibitors for each member of *ICH* are necessary. For the reasons discussed above, it seems likely that *crmA* can inhibit other members of *ICE/ced-3* family as well. These models can be tested directly by "knock-out" mutant mice in which a specific member of the *ICE/ced-3* family is mutated.

Experimental Procedures

Cloning and construction of plasmids

The mouse *nedd*-2 cDNA was isolated using embryonic mouse brain cDNA and the primer pairs specific for the 5' and 3' untranslated regions and the coding region. Primers *nedd2/1* (5'-CAACCCTGTAACTCTTGATT-3') and *nedd2/2* (5'-ACCTCTTTGGAGCTACCAGAA-3') were used for amplifying the 5' untranslated region. Primers *nedd2/3* (5'-CCAGATCTATGCTAACTGTCCAAGTCTA-3') and *nedd2/4*

(5'AAGAGCTCCTCCAACAGCAGGAATAGCA-3') were used for amplifying the *nedd-2* coding region. Primer *nedd2/5* (AGAAGCACTTGTCTCTGCTC) and *nedd2/6* (5'TTGGCACCTGATGGCAATAC-3') were used for amplifying the 3' untranslated region. 0.5 kb PCR product of *nedd-2* coding region was cloned into pBluescript plasmid vector to be used as a probe (Stratagene).

Two human fetal brain cDNA libraries and one mouse embryonic 11.5d cDNA library were screened with murine *nedd*-2 cDNA probe at low stringency and one mouse embryonic 11.5d cDNA library. The filters were hybridized in 5x SSPE, 30% formamide, 1x Denhardt's solution, 1% SDS at 42°C overnight and washed in 1x SSPE and 0.5% SDS, twice at room temperature and twice at 45°C (20 min). The human *Ich-1*$_S$ (pBSH30) was isolated from the positive clones using a *BamH*I-*Sal*I fragment of the murine *nedd*-2 cDNA, a 70 bp fragment which contains 52 bp of the 61 bp intron, as a probe under the same hybridization and washing conditions described above. The phage clones (pBSH37 for *Ich-1*$_L$, pBSH30 for *Ich-1*$_S$) were excised *in vivo* to obtain plasmids by an *in vivo* excision protocol (Stratagene). To construct expression constructs, PCR was performed using synthetic primers. H1 (5'-GATATCCGCACAAGGAGCTGA-3') and H2 (5'-CTATAGGTGGGAGGGTGTCC-3') were used for *Ich-1*$_L$ construction. H3 (5'-GATATCCAGAGGGAGGGAACGAT-3'), corresponding to sequences in the 5' region of *Ich-1*$_S$ cDNA and H4 (5'-GATATCAGAGCAAGAGAGGCGGT-3'), corresponding to the sequences in the 3' region of the first open reading frame (ORF) of *Ich-1*$_S$ were used for the first ORF of *Ich-1*$_S$ construction. H3 and H5 (5'-GATATCGTGGGAGGGTGTCCT-3'), corresponding to the sequences in the 3' region of the second ORF of *Ich-1*$_S$ were used for the second ORF of *Ich-1*$_S$ construction. pBSH37 and pBSH30 were used as templates where appropriate. The three PCR products were inserted into the *Eco*RV site of pBluescript II, and the inserts of these subclones, pβSIIh37, pβSIIh30.1, and pβSIIh30.2, were isolated by digestion with *Sma*I and *Kpn*I and cloned into

*Sma*I-*Kpn*I sites of *BSLacZ* (Miura, M., *et al.*, *Cell 75*:653-660 (1993)). *Not*I linkers were added to the *Kpn*I site by digesting with *Kpn*I, blunt-ending by T4 polymerase and ligating in the presence of excess *Not*I linker. These constructs, BSh37Z, BSh30Z1, and BSh30Z2, were digested with *Not*I and individually cloned into pβactstneoB (which uses the chicken β-actin promoter) (Miyawaki, A., *et al.*, *Neuron 5*:11-18 (1990)). The final plasmids were designated pβactH37Z, pβactH30Z1 and pβactH30Z2, respectively. pBabeH30 plasmid, used for establishing stable Rat-1 cell lines carrying *Ich-1*$_S$, was constructed by inserting the full length *Ich-1*$_S$ cDNA into the *Sal*I site of pBabe/puro vector (Morgenstern, J. P., *et al.*, *Nucl. Acids Res. 18*:3587-3596 (1990)).

To mutagenize Cys 303 to a Ser residue in the active domain of *Ich-1*$_L$ and Ala 352 to a Thr residue in the P10 subunit of *Ich-1*$_L$, primers containing mutant sites were synthesized as follows:

HM1 5'-ATCCAGGCCTCTAGAGGAGAT-3'
HM2 5'-ATCTCCTCTAGAGGCCTGGAT-3'
HM3 5'-TGCGGCTATACGTGCCTCAAA-3'
HM4 5'-TTTGAGGCACGTATAGCCGCA-3'

(HM1 corresponds with HM2 and HM3 corresponds with HM4. PCRs were performed in two steps. To make the Cys 303 to Ser mutation, in the first round of PCR, the fragments from the N-terminus to the mutation site of *Ich-1*$_L$ and from the mutant site to the C-terminus of *Ich-1*$_L$ were synthesized using two primer pairs, T3 and HM1, HM2 and T7, and PBSH37 as a template. In the second round of PCR, the two PCR fragments generated in the first reaction were used as templates and T7 and T3 were used as primers. Two such rounds of PCR generated a full length *Ich-1*$_L$ mutant. The other mutation was generated in a similar way, using T3 and HM3, HM4 and T7, for the Ala 352 to Thr mutation, as primers for the first PCR. The PCR products were inserted into the *Eco*RV site of pBluescript II and sequenced. The mutant cDNA inserts were cloned into expression vectors as described

above. The mutated clones were designated pβactH37ZCS and pβactH37ZAT.

Cell culture and functional studies

COS cells, Rat-1 cells, HeLa cells, and NG108-15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The day before transfection, cells were seeded at a density of about $2.5x10^5$ in each of the 6-well dishes. For each well, 0.7-1μg of the *lacZ* chimeric construct and 10μg of lipofectamine reagent were used according to a protocol from GIBCO BRL (Gaithersburg, MD). The cells were incubated for 3 hr in serum-free medium containing DNA and lipofectamine. Then an equal volume of growth medium containing 20% serum was added without removing the transfection mixture and incubation was continued for 24 hr. The expression of the chimeric gene in cells in culture was detected as previously described (Miura, M., *et al.*, *Cell 75*:653-660 (1993)).

To establish Rat-1 cell lines overexpressing *Ich-1$_S$*, pBabeH30 was transfected into Rat-1 cells using lipofectamine mediated gene transfer. Resistant cells were selected using 3μg/ml puromycin for about 10 days. Cells were assayed for expression of *Ich-1$_S$* by Northern blot analysis. To examine whether *Ich-1$_S$* can render Rat-1 cells resistant to apoptosis under conditions of serum deprivation, Rat-1 cells overexpressing *Ich-1$_S$*, untransfected control Rat-1 cells, transfected negative control Rat-1 cells, and Rat-1 cells overexpressing *bcl-2* or *crmA*, were seeded in 24-well dishes at $5x10^4$ cells in 500 μl of DMEM containing 10% FCS for 24 hr, washed once with serum-free DMEM, and transferred into 500 μl of serum-free DMEM. The cells were harvested at daily intervals and stained with 0.4% trypan blue for 5 min. at room temperature. The numbers of dead and living cells were counted using a haemocytometer.

Jurkat cells, THP.1 cells, and U937 cells were cultured in RPMI 1640 medium (GIBCO) with 10% fetal calf serum.

RNA analysis

The Multiple Tissue Northern (MTN) blots membrane of human fetal and adult tissues (CLONTECH) were probed using human *Ich-1*$_L$ cDNA or the intron of *Ich-1*$_S$ cDNA (for fetal tissue) in 5x SSPE, 10x Denhardt's solution, 50% formamide, 2% SDS, and 100μg/ml salmon sperm DNA at 42°C overnight. The blots were washed twice in 2x SSPE and 0.05% SDS at room temperature, and twice in 0.1x SSPE, and 0.1% SDS for 20 min. at 50°C.

The Multiple Tissue Northern (MTN) blot membrane of human fetal tissue was first probed with a 1.3 kb fragment from the insert of pBSH37, which hybridizes to both *Ich-1*$_L$ and *Ich-1*$_S$. The blot was exposed for two days and developed. Then the blot was stripped by boiling the filter in H_2O twice for 20 min. After stripping, the filter was re-exposed for three days to ensure that the stripping was complete. Then the filter was re-hybridized with the 70 bp *Bam*HI-*Sal*I fragment derived from mouse *nedd-2* gene. This fragment contains 52 bp of the 61 bp intron (which is identical to the human *Ich-1*$_S$ intron; the remaining 18 bp are from an exon and 5 out of the 18 bp are different from the human *Ich-1* sequence). The Northern blot of THP.1 cells and U936 cells was carried out under the same conditions. To detect *Ich-1* expression in Rat-1 cells, hybridization was carried out in 25% formamide and under otherwise identical conditions.

Cloning of murine Ich-1

A murine *Ich-1* cDNA was cloned in two steps by PCR. A 5' murine *Ich-1* cDNA fragment was amplified using a primer derived from the pBSH37 5' end sequence (5' ATTCCGCACAAGGAGCTGATGGCC 3') and a primer from the mouse *nedd-2* after the active site sequence

(5′ GCTGGTCGACACCTCTATC 3′) using a mouse embryo cDNA library as template (a gift from D. Nathan). The resulting 945bp fragment was cloned into the *Eco*RV site of plasmid pBSKII (Stratagene) and sequenced. A 3′ murine *Ich-1* cDNA fragment was amplified using a human *Ich-1* primer further downstream (5′ CAAGCTTTTGATGCCTTCTGTGA 3′ and a *nedd-2* primer downstream from the coding region (5′ CTCCAACAGCAGGAATAGCA 3′). The resulting fragment was also cloned in pBSKII and sequenced. The two fragments were then joined together using an unique *Sal*I site at nucleotide 930 from the beginning of the coding region.

Cloning of chicken Ich-1

A chicken *Ich-1* cDNA fragment was obtained using murine *Ich-1* degenerate primers. The 5′ primer was from murine *Ich-1* nucleotide 241 to 268 bp:
5′GC(GATC)TT(TC)GA(TC)GC(GATC)TT(TC)TG(TCG)GA(AG)GC3′.
The 3′ primer was from murine *Ich-1* nucleotide 883 to 908 bp:
5′CA(GATC)GC(CT)TG(TAG)AT(AG)AA(AG)AACAT(CT)TT(GATC)GG3′.
The resulting DNA fragment was used as a probe to screen a chicken embryonic library (Clontech) by high stringency hybridization.

Quantitative PCR analysis

mRNA was isolated using the MicroFast mRNA isolation kit from Invitrogen. 1 to 2 μg of mRNA was used from reverse transcription by random priming (Invitrogen) using MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (Invitrogen). The primers used to amplify murine *Ich-1*: 5′ primer (5′ ATGCTAACTGTCCAAGTCTA 3′) and 3′ primer (5′ GTCTCATCTTCATCAACTCC 3′). The primers used to amplify human *Ich-1*: 5′ primer (5′ GTTACCTGCACACCGAGTCACG 3′) and 3′ primer

(5' GCGTGGTTCTTTCCATCTTGTTGGTCA 3'). The primers used to amplify *hICE*: 5' primer (5' ACCTTAATATGCAAGACTCTCAAGGAG 3') and 3' primer (5' GCGGCTTGACTTGTCCATTATTGGATA 3'). Mouse β-actin primers were used as controls to amplify a 350 bp actin fragment from mouse and human tissue. 5' β-actin primer: 5' GACCTGACAGACTACCTCAT 3'. 3' β-actin primer: 5' AGACAGCACTGTGTTGGCAT 3'. The following conditions were used for the PCR reactions: 1 x reaction buffer (Promega), 1.5 mM $MgCl_2$, 200 μM dNTP, 2 μM each primers, 1 unit of Taq DNA polymerase (Promega) in a total volume of 50 μl. The DNA was denatured for 4 min at 94°C prior to 26 PCR cycles (94°C, 1 min/55°C, 1 min/72°C, 2 min). In some of the experiments, Vent polymerase (Biolab) was used.

In vitro transcription and translation of Ich-1$_S$

To determine which open reading frame of *Ich-1$_S$* was expressed, pBluescript plasmid containing *Ich-1$_S$* (pBSH30) was linearized at the 3' multiple cloning site with *Xho*I, purified, and transcribed with T3 RNA polymerase for 2 hr at 37°c using a protocol from Stratagene. The plasmid was also linearized at the 5' multiple cloning site with *Not*I, purified, and transcribed with T7 polymerase as an antisense control. The resulting runoff transcripts were extracted with phenol-chloroform and ethanol precipitated. *In vitro* translation was performed with rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine for 1 hr. at 30°C. 5μl lysate was mixed with equal volume of 2xSDS gel loading buffer and subjected to SDS-polyacrylamide gel electrophoresis (12%). The gel was dried and exposed to X-ray film.

Example 6

Experimental Procedures

Cells and tissue culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HeLa cells were transfected with pHD1.2 *crmA* expression vector (Gagliardini, V. *et al.*, *Science 263*:826-828 (1994)) by calcium phosphate precipitation and two days after transfection, 600 μg/ml of G418 (Gibco) was added for selection. Resistant colonies were cloned by limiting dilution. Dosage response of HeLa and HeLa/crmA cells to TNF-α treatment was tested as follows. Cells were seeded in DMEM plus 10% fetal calf serum in a 24-well plate at a density of $4x10^4$ cells per well. After overnight incubation, the cells were washed twice with serum-free DMEM. Drugs were then added to a total volume of 0.2 ml of serum-free DMEM and the cells were incubated for 24 hours. Cells were then trypsinized and dead cells scored on a hemocytometer by trypan blue exclusion (Sigma, St. Louis, MO). At least 200 cells were scored per well. Each concentration was tested in duplicate each time.

Western blotting

Cells were lysed in SDS sample buffer and cell lysates were subjected to 15% SDS-PAGE. After electroblotting the proteins to an Immobilon nylon membrane (Millipore), the membrane was blocked with 4% nonfat milk in 25 mM Tris-HCl pH7.5, 150 mM NaCl, 0.2% Tween (TBST). The membrane was incubated with anti-crmA antibody (Gagliardini *et al.*, *Science 263*:826-828 (1994)) (5 μg/ml) for 1 hour at room temperature and then washed five times with TBST. The membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1/1000 dilution, Amersham) for 30 minutes and washed five

times with TBST. crmA protein was detected with an ECL detection kit (Amersham).

DNA transfection

One day before transfection, cells were seeded at a density of about 2 x10^5 per well in 6-well dishes. For each well, 1 μg of plasmid DNA and 10 μg of lipofectamine reagent was added according to a protocol from Gibco BRL. Cells were incubated for 3 hours in serum-free medium containing DNA and lipofectamine, and then medium was changed to DMEM containing 10% FBS and incubation was continued for 24 hours. The expression of chimeric gene was detected as previously described (Muira *et al.*, *Cell 75*:653-660 (1993)).

Detection of IL-1β production from HeLa cells

HeLa cells were grown overnight in medium containing 10% fetal calf serum and then the medium was changed to a serum-free DMEM with or without drugs. After 24 hours, cells were scraped off and precipitated. Conditioned medium was collected, dialyzed against distilled water at 4°C overnight, lyophilized, and the residue dissolved in distilled water. Cell precipitates were extracted with extraction buffer (20 mM HEPES-NaOH pH7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EDTA, 10 μg/ml PMSF, 10 μg/ml E64, 2 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5 μg/ml aprotinin, 1% NP-40). Insoluble materials were removed by centrifugation. Proteins were separated by 15% SDS-PAGE and IL-1β was detected by immunoblotting using anti-human IL-1β antibody (1/300 dilution, Calbiochem).

Results

Establishment of crmA-expressing HeLa cells

To test the hypothesis that activation of *ICE* is responsible for TNF-α induced apoptosis, the inventors first established HeLa cell lines constitutively expressing cowpox virus crmA protein. This protein is a viral serpin that can specifically inhibit ICE activity (Ray, C.A. *et al.*, *Cell* 69:597-604 (1992)).

HeLa cell clones expressing crmA were analyzed by Western blot analysis with affinity-purified anti-crmA. HeLa cells were transfected with crmA expression vector and selected for G418 resistance as described in "Experimental Procedures" above. Six different G418 resistant HeLa cell clones were analyzed. As a positive control, cell lysate of a Rat-1 cell clone expressing *crmA* (Miura, H. *et al.*, *Cell* 75:653-660 (1993)) was applied to the gel. Several HeLa cell clones stably expressing *crmA* were established.

Overexpression of Ice/ced-3 family gene in HeLa/crmA cells

The cell lines were tested for resistance to cell death induced by *ICE* overexpression. The overexpression of *crmA* in HeLa cells suppressed *ICE*-induced cell death. HeLa cells constitutively expressing *crmA* were transfected with a chimeric expression vector expressing both the *lacZ* and the *mICE* gene (pβactM10Z). Transfection is described above in "Experimental Procedures". One of the HeLa/crmA clones expressing high levels of crmA could efficiently suppress *ICE* induced cell death. A clone expressing *crmA* at low levels also could efficiently suppress the *ICE* induced cell death under the same conditions (viability 69.8 ± 1.2%, Table 4.)

Table 4. Prevention of apoptosis by CrmA

	% round blue cells	
Expression cassettes	HeLa	Hela/CrmA
pactβGal'	3.2 ± 0.8	1.1 ± 0.4
pβactM10Z	85.5 ± 3.0	46.9 ± 7.0
pβactH37Z	91.4 ± 4.9	87.5 ± 3.0
Drug treatments	% dead cells	
control	1.5 ± 0.3	5.5 ± 1.8
CHX	2.9 ± 0.7	3.8 ± 0.5
TNF	2.7 ± 1.2	2.8 ± 1.0
CHX + TNF	68.2 ± 1.9	9.7 ± 1.2

Cells were transfected and stained as described herein. Plasmid pactβGal' is a control *LacZ* gene expression vector and plasmid pβactM10Z is a mouse *Ice/lacZ* chimeric gene expression vector (Muira, M. *et al.*, *Cell 75*:653-66 (1993)). Plasmid pβactH37Z is a *Ich-1/lacZ* chimeric gene expression vector (Lin, W. *et al.*, *Cell 78*:739-750 (1994). The data (mean ± SEM) shown are the percentage of round blue cells among total number of blue cells counted. To see the effects of CHX (20 μg/ml) and the TNF-α (5 ng/ml), cells were treated with drugs for 24 h and cell viabilities were measured by typan blue dye exclusion. The data (mean ± SEM) shown are the percentage of dead cells. The data were collected from at least three independent experiments.

CrmA is a potent and highly specific serpin for ICE. However, cell death induced by overexpression of *ced-3* is poorly suppressed by crmA (Miura, M. *et al.*, *Cell 75*:653-660 (1993)). *Ich-1 (nedd-2)* has been described above as third member of the *ICE/Ced-3* gene family (*see* also, Kumar, S. *et al.*, *Genes Dev. 8*:1613-1626 (1994)). As with *ICE*, overexpression of *Ich-1* induces Rat-1 and HeLa cell death efficiently. However, *Ich-1* induced cell death is weakly suppressed by overexpression of crmA in HeLa cells (Table 4) and Rat-1 cells. Thus, crmA does not appear to be a general inhibitor of *ICE/Ced-3* family protease.

Suppression f TNFα-induced apoptosis by crmA

The effect of *crmA* expression on TNF-induced apoptosis was tested. TNF induced cytotoxicity was suppressed by overexpression of *crmA*. HeLa cells or HeLa/crmA cells were treated with cycloheximide (CHX) (20 μg/ml, Sigma), TNF-α (5ng/ml, Sigma), or a combination of both drugs. Cells were photographed 24 hours after drug treatment. Control HeLa and HeLa/crmA cells were tested for the ability to resist increasing amounts of TNF-α in the presence of 10 μg/ml CHX (Figure 18). In the presence of CHX, TNF-α efficiently induced HeLa cell death (White, E. *et al.*, *Mol. Cell. Biol.* *12*:2570-2580 (1992)). Under the same conditions, HeLa cells expressing crmA at high levels are resistant to the TNF-α cell death stimulus (Table 4). A clone of HeLa/crmA cells that expresses lower levels of crmA was also resistant under the same conditions (% dead cells = 30.2±1.2%). The dose response of *crmA*-expressing HeLa cells to increasing amounts of TNF-α in the presence of 10 μg/ml of CHX was tested. HeLa/crmA cells are resistant to 0.01pg/ml to 100ng/ml of TNF-α (Figure 16). After a 24 hour incubation in the presence of 100ng/ml TNF-α, 83% of the control HeLa cells died compared to 23% of HeLa/crmA cells.

Activation of endogenous ICE after TNF stimulation

The inventors have detected the expression of both *ICE* and *Ich-1* in HeLa cells (Lin, W. *et al.*, *Cell 78*:739-750 (1994)). Since *Ich-1*-induced cell death is only weakly suppressed by crmA and crmA appears to be very effective in preventing cell death induced by TNF and CHX treatment, the *ICE*-mediated cell death pathway may be activated by TNF stimulation and may play a role in HeLa cell death. If this is the case, TNF stimulation should activate endogenous *ICE* in HeLa cells.

Pro-IL-1β is the only known endogenous substrate for *ICE*. Active ICE is an oligometric enzyme with p20 and p10 subunits (Thornberry, N.A.

et al., *Nature 356*:768-774 (1992); Cerretti, D.P. *et al.*, *Science 256*:97-100 (1992)). These subunits are derived from a p45 precursor form of ICE (Thornberry, N.A. *et al.*, *Nature 356*:768-774 (1992); Cerretti, D.P. *et al.*, *Science 256*:97-100 (1992)). If *ICE* is activated after TNF stimulation, the endogenous 33kd pro-IL-1β should be processed and mature 17.5kd IL-1β secreted into the medium.

To detect mature IL-1β, conditioned medium was collected from HeLa cells with or without TNF stimulation. The processing of pro-IL-1β was analyzed by Western blot. Processing of IL-1β was observed only after induction of apoptosis by TNF-α/CHX in HeLa cells. The following samples were compared: purified mature human IL-1β, cell lysates (10 μg protein/lane), conditioned medium (5 μg/lane), serum-free controls, LPS (10 μg/ml, Sigma) treatment, cycloheximide (20 μg/ml) and TNF (5 ng/ml).

The procedure for detecting IL-1β is described above under "Experimental Procedures". Cell viabilities were measured by trypan blue dye exclusion (97.4 $\pm$ 1.5% for serum free control, 97.4 $\pm$ 0.2% for LPS treatment, 56.3 $\pm$ 2.2% for CHX/TNF-α treatment). The data showed that mature IL-1β was only observed after induction of apoptosis by TNF. These results strongly suggest that TNF stimulation induces apoptosis by activation of an *ICE*-dependent cell death pathway.

Discussion

The inventors have demonstrated herein that overexpression of *ICE* induces Rat-1 cells to undergo apoptosis (Miura, M. *et al.*, *Cell 75*:653-660 (1993)) and expression of *crmA* can prevent chicken DRG neurons from cell death induced by trophic factor deprivation (Gagliardini, V. *et al.*, *Science 263*:826-828 (1994)). These results show that *ICE* has the ability to induce cell death and that inhibition of *ICE* activity can prevent programmed cell death. However, the results did not show that *ICE* was, indeed, activated during programmed cell death. Using pro-IL-1β processing as an indicator,

the inventors have demonstrated that *ICE* is activated when HeLa cells are induced to die with TNF-α and CHX.

ICE has unique substrate specificity which requires an Asp in the P1 position (Sleath *et al.*, *J. Biol. Chem* 265:14526-14528 (1990)). Only two eukaryotic proteases are reported to cleave after the Asp. The other is granzyme B, a serine protease in the cytotoxic granules of killer T lymphocytes (Odake *et al.*, *Biochemistry 30*:2217-2227 (1991)). In THP.1 cells and HeLa cells, expression of both *ICE* and *Ich-1* were detected (Wang *et al.*, *Cell 78*:739-750 (1994)). However, affinity labeling of THP.1 cell lysates with a competitive, irreversible ICE inhibitor, biotinylated tetrapeptide (acyloxy) methyl ketone, resulted in labeling only of ICE (Thornberry *et al.*, *Biochemistry 33*:3934-3940 (1994)). This suggests that ICE is the only enzyme to cleave proIL-β in the human monocytic cell line, THP.1. The present studies show that *crmA* cannot prevent cell death induced by *Ich-1* in HeLa cells.

How CHX potentiates the TNF cytotoxicity in non-transformed cells is unclear. Most of the cell lines, including HeLa cells, NIH3T3 cells, and TA1 cells, are not killed by TNF alone, but are killed by the combined action of TNF and CHX (Reid *et al.*, *J. Biol. Chem. 264*:4583-4589 (1991); Reid *et al.*, *J. Biol. Chem. 266*:16580-16586 (1991)). TNF-α is a pleiotrophic cytokine which may induce more than one cellular response in a single cell. The presence of CHX may inhibit the synthesis of certain signaling molecules and thus, potentiate the killing activity of TNF. Alternatively, CHX may simply inhibit the synthesis of a general cell survival factor(s) and thus, allow cells to become more sensitive to TNF cytotoxicity.

HeLa cells express predominantly p55 TNF receptor, thought to be responsible for cell death signalling (Engelmann *et al.*, *J. Biol. Chem. 265*:14497-14504 (1990); Thoma *et al.*, *J. Exp. Med. 72*:1019-1023 (1990)). TNF p55 receptor triggers the activation of phospholypase A2, protein kinase C, sphingomyelinase, phosphatidylcholine-specific phospholypase C, and NF-kB (Weigmann *et al.*, *J. Biol. Chem. 267*:17997-18001 (1992); Schutze *et al.*,

Cell 71:765-776 (1992)). In TNF p55 receptor knockout mice, TNF-mediated induction of NK-kB is prevented in thymocytes (Pfeffer *et al.*, *Cell 73*:457-467 (1993)). TNF p55 receptor knockout mice were resistant to lethal doses of either lipopolysaccharides or *S. aureus* enterotoxin B. This suggests that TNF p55 receptor mediated hepatocyte necrosis (Pfeffer *et al.*, *Cell 73*:457-467 (1993)). After the stimulation of HeLa cells by TNF-α/CHX, not all the proIL-1β was converted into mature IL-1β. ICE activity is likely to be tightly controlled within the cells. A small amount of active ICE may be sufficient for induction of apoptosis. Even in the mature IL-1β-producing monocytic cell line, THP.1, most of the ICE is the p45 inactive form. In non-LPS-stimulated THP.1 cells, only 0.02% of ICE is in the active form. In stimulated cells, the maximum amount of active ICE is less than 2% of total ICE (Ayala *et al.*, *J. Immunol. 153*:2592-2599 (1994)). THP.1 cells may have some protective mechanism to prevent the activated ICE from inducing apoptosis. LPS induces the synthesis of a large amount of pro-IL-1β, which may, in fact, confer protection on THP.1 cells, because substrates usually are good competitive inhibitors of enzymes.

In addition to the secretion of mature IL-1β, there was a significant drop of the proIL-1β level in the cell lysates prepared from TNF-α/CHX-treated cells. This could be the result of secretion of mature IL-1β, inhibition of biosynthesis of proIL-1β, or increase of proteolytic activity within the dying cells. If, however, the inventors' hypothesis is correct (i.e., that pro-IL-1β does act as a competitive inhibitor of ICE), a reduction in the level of pro-IL-1β would in fact represent a further reduction in cellular defense against apoptosis and could be one of the reasons that CHX can increase the cytotoxicity of TNF-α.

TNF is a central component in the mammalian host inflammatory response (Tracey, K.G. *et al.*, *Ann. Rev. Cell. Biol. 9*:317-343 (1993)). In models of septic shock, injection of endotoxin (LPS) rapidly induces TNF, IL-1 and IL-6 (Ford, Y. *et al.*, *J. Exp. Med. 170*:1627-1633 (1989)). Under these conditions, the secretion of IL-1β appears to be dependent upon TNF

because passive immunization with TNF monoclonal antibodies during endotoxemia *in vivo* attenuates the appearance of IL-1β (Fong, Y. *et al.*, *J. Exp. Med. 170*:1627-1633 (1989)). The results herein show that TNF plays a role in activating *ICE*, the key enzyme in processing IL-1β.

Expression of mitochondrial manganese superoxide dismutase has been shown to promote the survival of tumor cells exposed to TNF (Hirose, K. *et al.*, *Mol. Cell. Biol. 13*:3301-3310 (1993)). This suggests that the generation of free radicals plays a role in cell death induced by TNF. There are several reports that TNF cytotoxicity is related to the generation of free radicals and lipid peroxides (Hennet, T. *et al.*, *Biochem. J. 289*:587-592 (1993); Schulze-Osthoff, K. *et al.*, *J. Biol. Chem. 267*:5317-5323 (1992)). If that is the case, *ICE* may be activated directly or indirectly by free radicals.

The death of Hela cells induced by TNF is also suppressed by *bcl-2* overexpression (the viability is 83.7 $\pm$ 2.3% under the experimental conditions described for Table 4). Bcl-2 has been suggested to have the ability to either inhibit the production of free radicals (Kane, D.J. *et al.*, *Science 262*:1274-1277 (1993)) or prevent free radicals from damaging cells (Hockenberry, D.M. *et al.*, *Cell 75*:241-251 (1993)). Thus, in HeLa cells, *bcl-2* and *crmA* may suppress cell death induced by TNF through a single biochemical pathway of programmed cell death.

Fas/Apo (ref)-antigen is a member of TNF receptor family (Itoh, N. *et al.*, *Cell 66*:233-243 (1991)). Apoptosis can be induced by stimulation of Fas-antigen by anti-Fas/anti-Apo antibody (Yonehara, S. *et al.*, *J. Exp. Med. 169*:1747-1756 (1989)) or Fas-ligand (Suda, T. *et al.*, *Cell 75*:1169-1178 (1993)), a type II transmembrane protein homologous to TNF. Cell death signalling after the stimulation of Fas-antigen is largely unknown. However, Fas mediated cell death is protected by overexpression of *E1b* (Hashimoto, S. *et al.*, *Intern. Immun. 3*:343-351 (1991)) or *bcl-2* (Itoh, N. *et al.*, *J. Immun. 151*:621-627 (1993)). The data presented herein suggest that stimulation of Fas-antigen may also activate *ICE/ced-3* cell death pathway.

What Is Claimed Is:

1. A method of preventing programmed cell death in vertebrates comprising the step of inhibiting the enzymatic activity of interleukin-1β converting enzyme.

2. The method of claim 1, wherein said enzymatic activity is inhibited by an interleukin-1β converting enzyme-specific antiprotease.

3. The method of claim 2, wherein said antiprotease is encoded by the *crmA* gene.

4. A method of promoting programmed death in vertebrate cells by increasing the enzymatic activity of interleukin-1β converting enzyme in said cells.

5. The method of claim 4, wherein said vertebrate cells are cancer cells.

6. The method of claim 5, wherein said cancer cells overexpress the oncogene *bcl-2*.

7. A substantially pure gene which is preferentially expressed in thymus and placental cells and which encodes a protein causing programmed cell death.

8. The gene of claim 7, wherein said protein has the amino acid sequence shown in Figure 6.

9. The gene of claim 8, wherein said gene has the cDNA sequence shown in Figure 6.

10. An expression vector having the gene of either claim 8 or claim 9.

11. A host cell transformed with the vector of claim 10.

12. A substantially pure protein wherein said protein is preferentially expressed in thymus or placental cells and which causes the death of said cells.

13. The protein of claim 12, wherein said protein has the amino acid sequence shown in Figure 6.

14. A functional derivative of the protein of claim 13.

15. A method of promoting programmed cell death in thymus or placental cells comprising the step of increasing the activity of the protein of claim 7.

16. A substantially pure DNA molecule comprising a cDNA sequence selected from the group consisting of the cDNA sequence shown in Figures 10A and 10B.

17. An expression vector having the DNA of claim 16.

18. A host cell transformed with the vector of claim 17.

19. A substantially pure protein comprising an amino acid sequence selected from the group consisting of the amino acid sequence shown in Figures 10A and 10B.

20. A functional derivative of the protein of claim 19.

21. A substantially pure DNA molecule comprising the cDNA sequence shown in Figure 14.

22. An expression vector having the DNA of claim 21.

23. A host cell transformed with the vector of claim 22.

24. A substantially pure protein comprising the amino acid sequence shown in Figure 14.

25. A functional derivative of the protein of claim 24.

26. A method of regulating interleukin-1β converting enzyme by regulating the levels or activity of tumor necrosis factor.

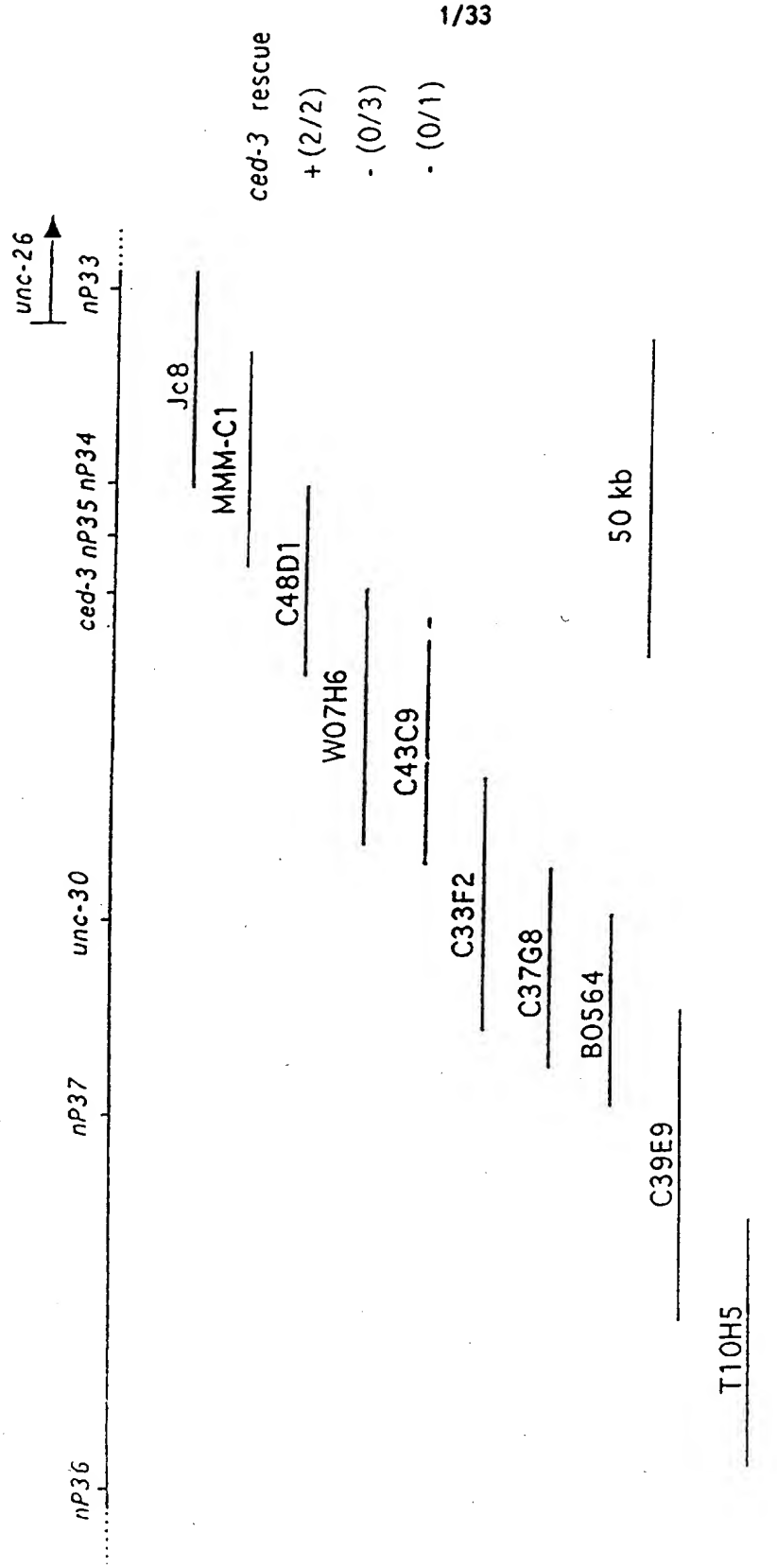
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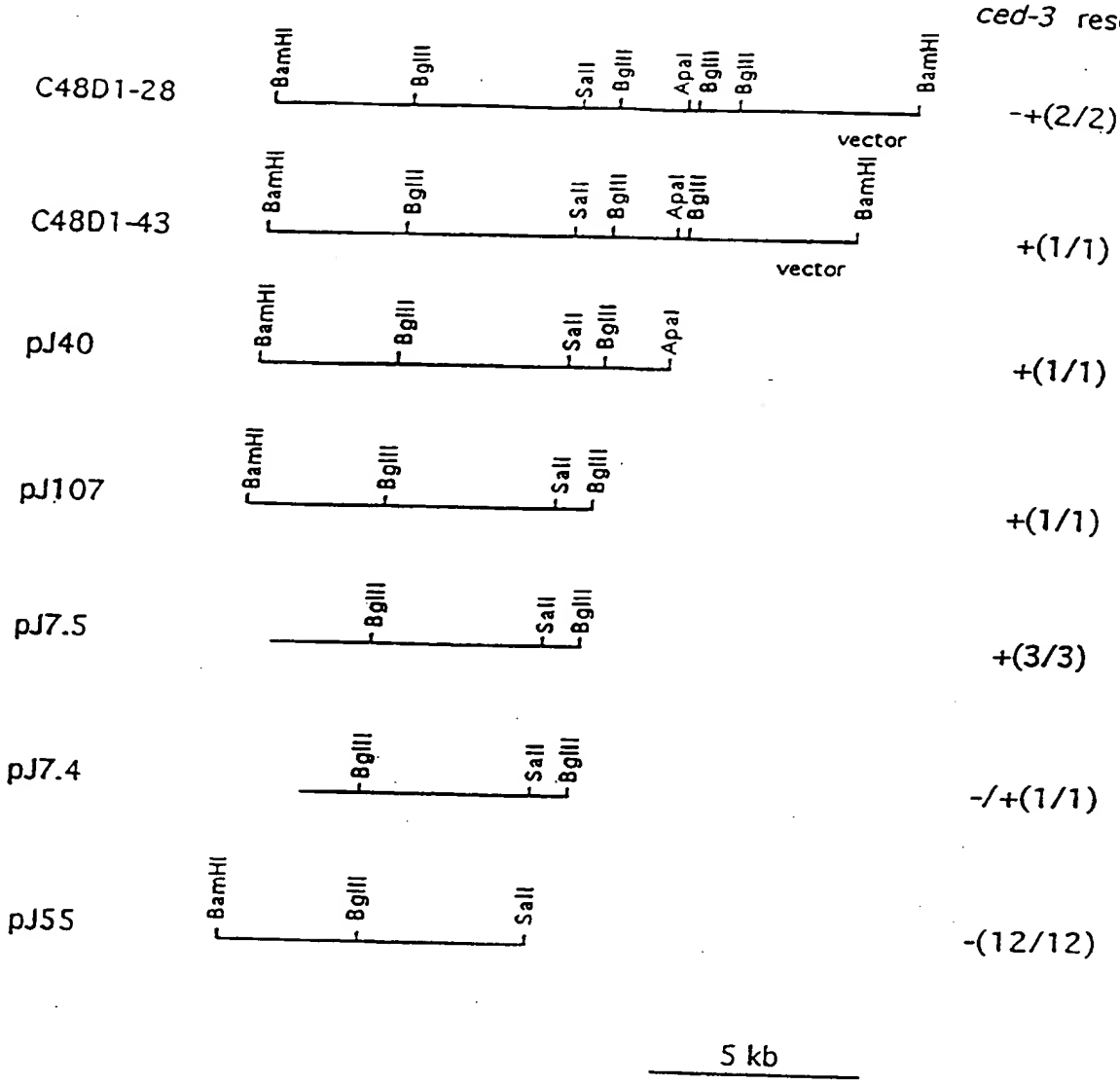
Figure 1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pJ107
BamHI
BglII
SalI
BglII
+(1/1)
pJ7.5
BglII
SalI
BglII
+(3/3)
pJ7.4
BglII
SalI
BglII
-/+(1/1)
pJ55
BamHI
BglII
SalI
-(12/12)
5 kb

FIGURE 1A

Figure 2

```
Repeat 1

                    1       10        20        30        40        50        60        70
ced-3(1a,for)       GTATTAA-GGAATCACAAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGG-CAAAATATCTCGTAGCG
ced-3(1a,rev)       ----------------AAAATTCAGAGAATGCGTATTACAGTC-ATATTTGGCGCGCAAAATATCTCGTAGCT
ced-3(1b,for)       ----------------AAATTCTGAGAATGCGCATTACTCAACATATTTGACGCGC-AAATATCTCGTAGCG
ced-3(1b,rev)       ----------------------------------------------------TATCTTGAAGCG
fem-1(for)          GTATTAC-GGCAAGAAATAATTATGAGAATGCCTATTGCGCACCATAGTTGACGCGCAAAATATCTCGTAGCG
fem-1(rev)          GTATAAC-GGTAACACACAATTCTGAGAATGCGTATTGCACAACACATTTGACGCGCAAAATATCTCGTAGCG
hlh-1(for)          CTATTAC-GGGAGTACAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGCGCAAAATATTTCGTATCG
hlh-1(rev)          ----------GGGAGCACAAAATTCTGACTATGAGAAT-GCGTATAA----------GCACAAAATATTTCGTAGCG

consensus           -TAT-A--GG-A--A-A-AATT--GA--ATG---A-T-C-----A-A-TTG-CG--CAAAATAT-T-G-A-C-

                      80        90        100       110       120       130       140
ced-3(1a,for)       AAAACTACAGTAATTCTTTAAATGACTACTGTAGCGC-----TTGTGTCGA-TTTACGGGCTCAATT---------
ced-3(1a,rev)       AGAACTACAGTAATCCTTTAAATGACTACTGTAGCG-;-----TTGTGACGA-TTTACGGGTTATCAAAATTCGAAA
ced-3(1b,for)       AAAA-TACAGTAACCCTTTAAATGACTATTGTAG----------TGTCGA-TTTACGGGC--TCGATTTTCGAAA
ced-3(2b,rev)       AAAACTACTGTAACTCTTTAAAAGAGTACTGTAGCGC-----TGGTGTCTG-TTTACGGAAATAATT---------
fem-1(for)          AAAACTACAGTAACTCTTTGAATGACTACTGTAGCGC-----TTGTTTCGA-TTTACGGGCTCGTT----------
fem-1(rev)          AAAACTACAGTGATTCGCTGAATGAATACGGTAGGGTCG----------------------------------------
hlh-1(for)          AAAACTACAGTAATTCGTTTATTGGCTACTGT-GCG----------TGTTGA-TTTACGGGC----------------
hlh-1(rev)          AAAACTACAGTAATTTGTCAAGGACTACTGTAGCTAGCG-CTTGTGTCGA-TTTACGGAGC-TCGATTTT------

consensus           A-AACTAC-GT-A--------A--G--TA--GTAG----------T-GT--------TTTACGG-----------TT-GAAA
```

FIGURE 2A(i)

Repeat 2

```
                1       10        20        30        40        50        60        70        80
ced-3(for)      TCATTCAAGATATGCTTATTAACACATATAATTATCATTAATGTGAATTTCTTGTAGAAATTTTGGGCTTTCGTTCTAG
ced-3(rev)      TCATTCAAGATATGCTTATTAACATCTATAATTATCAATAAAGGTAATATCTTGAAGAAATTTTGG--TTTTCGCTCTAA

consensus       TCATTCAAGATATGCTTATTAACA--TATAATTATCA-TAA-G--AAT-TCTTG-AGAAATTTTGG--TTTTCG-TCTA-

                       90       100       110       120       130       140       150       160
ced-3(for)      TATGCTCTACTTTTGAAATTGCTCAACGAAAAAAT-----------CATGTGCTTTGTTCATATGAATGACGAAAAATA
ced-3(rev)      TATTCTCTACTTTTTAAGTTGCTCAACGAAAAAATAATGGGGTTAATCATGTG----------------ATGTTGAAAAATA

consensus       TAT-CTCTACTTTT-AA-TTGCTCAACGAAAAAAT-----------CATGTG----------------ATG--GAAAAATA

                       170       180       190       200       210       220       230       240
ced-3(for)      GCAA-----T-TTTTTATATATTTT-CCCCTATTCATGTGTGCAGAAAAATAGTAAAAAAGGCATGCATTTTT-:....
ced-3(rev)      CAAAAATGTATTTTAATACATTTTCCCCCTATTCAT-TTGTGCAGAAAAGT-GTAAAAAACGCATGCATTTTTTACAT

consensus       --AA-----T-TTTT-ATA-ATTTT-CCCCTATTCAT-TTGTGCAGAAAA-T-GTAAAAAA-CGCATGCATTTTT-....

                       250       260       270       280       290
ced-3(for)      ----CGACA-TTTTTTACATCGAACGACAGCTCACTTCACATGCTGAAGACGAGAGACG
ced-3(rev)      TATTCGACATTTTTTTACATCGACCGAGATCCCATTTCACATGCTGAAGACGAGAGACG

consensus       ----CGACA-TTTTTTACATCGA-CGA-A-C-CA-TTCACATGCTGAAGACGAGAGACG
```

FIGURE 2A(ii)

FIGURE 2A(iii)

Repeat 4

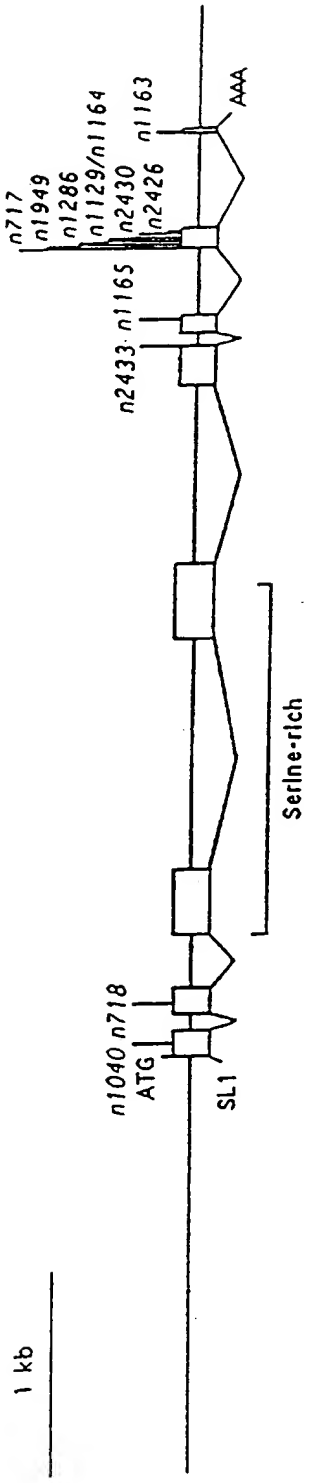
```
                1       10        20        30        40        50        60        70
ced-3           AACCAATCAGCATCGTCGATCTCCCGCCCACTTCATCGGATTGGTTTGAAAGTGGCGGAGTGAATTGCTGATTGGTC
lin-12          AACCAATTAGCGACTTCGGAATTTCCATACTTAATCTGATTGGTTGAAGAATGGGCAGAGCGAATTGCTGATTGGCC

consensus       AACCAAT-AGC--C-TCG---T---C--ACTT-AIC-GATTGGTT--A-A-TGGGC-GAG-GAATTGCTGATTGG-C
```

FIGURE 2A(iv)

Repeat 5

	10	20	30	40	50	60
ced-3(for)	TTTTAAG-GACACAGAAAAATAGGCAGAGGCTCCTTTTGCAAGCCTGCCGCGCGGTCAACC					
ced-3(rev)	TTTCAAGCCGCACAGAAAAAGAGGCGGAGCCGTCGTTTTGCAAACTTGCCGCGCGCCAACC					
consensus	TTT-AAG---CACAGAAAAA-AGGC-GAG--TC-TTTTGCAA-C-TGCCGCGCG-CAACC					

FIGURE 2A(v)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n1164
n1129/n1286
n1949
n717
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n2433
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ATG
SL1
1 kb

FIGURE 2B

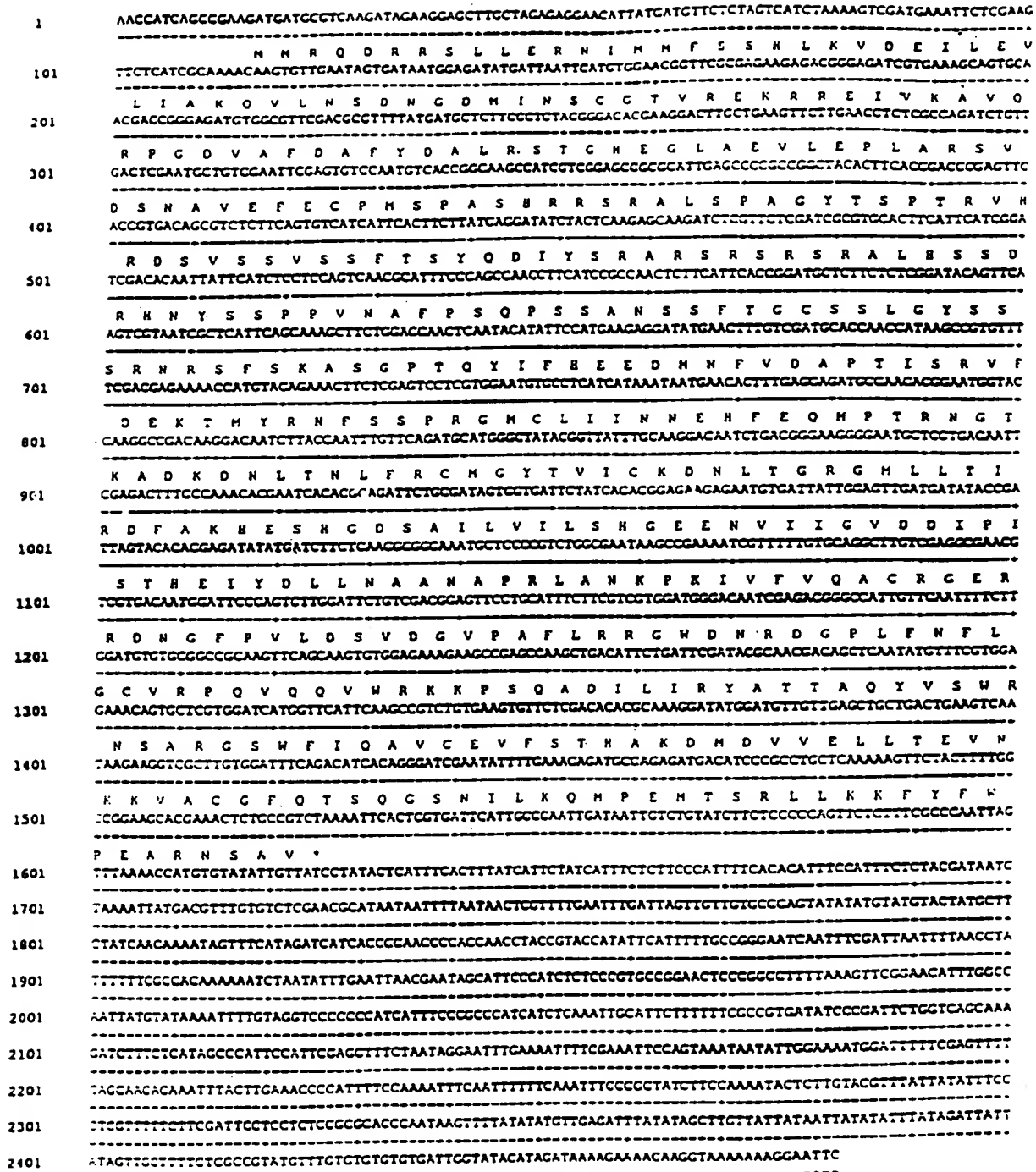
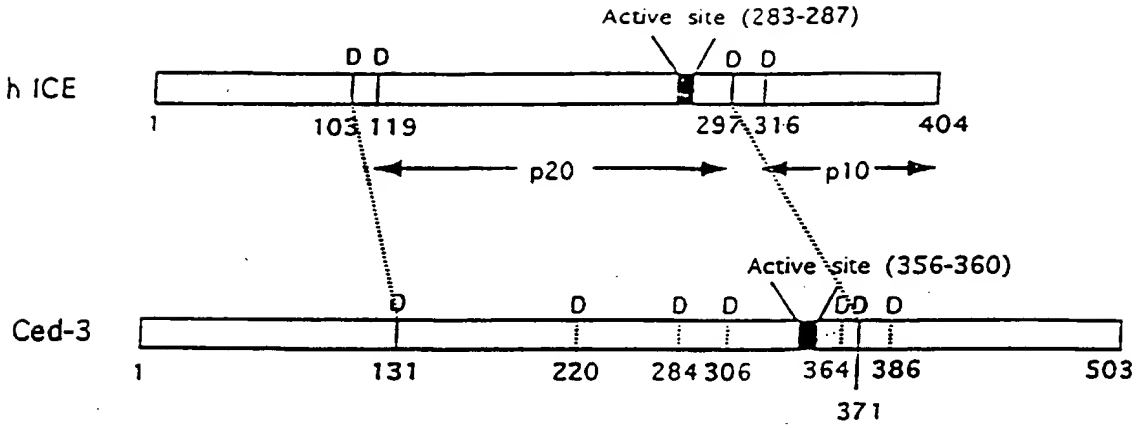


FIGURE 2C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h ICE
D D
D D
1
103 119
297 316
404
p20
p10
Active site (356-360)
Ced-3
D
D
D
D
D D
D
1
131
220
284 306
364
386
503
371

FIGURE 3

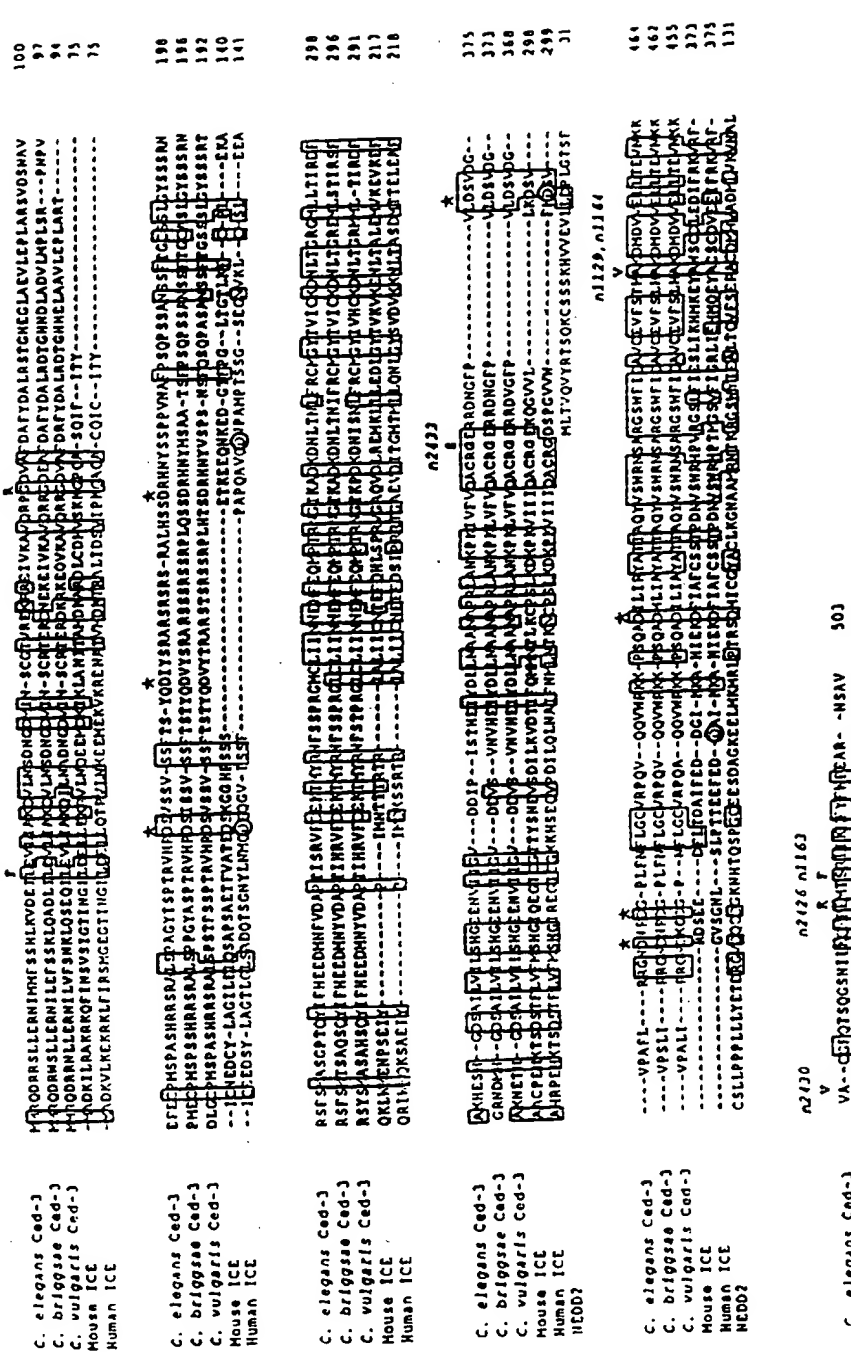

FIGURE 3A

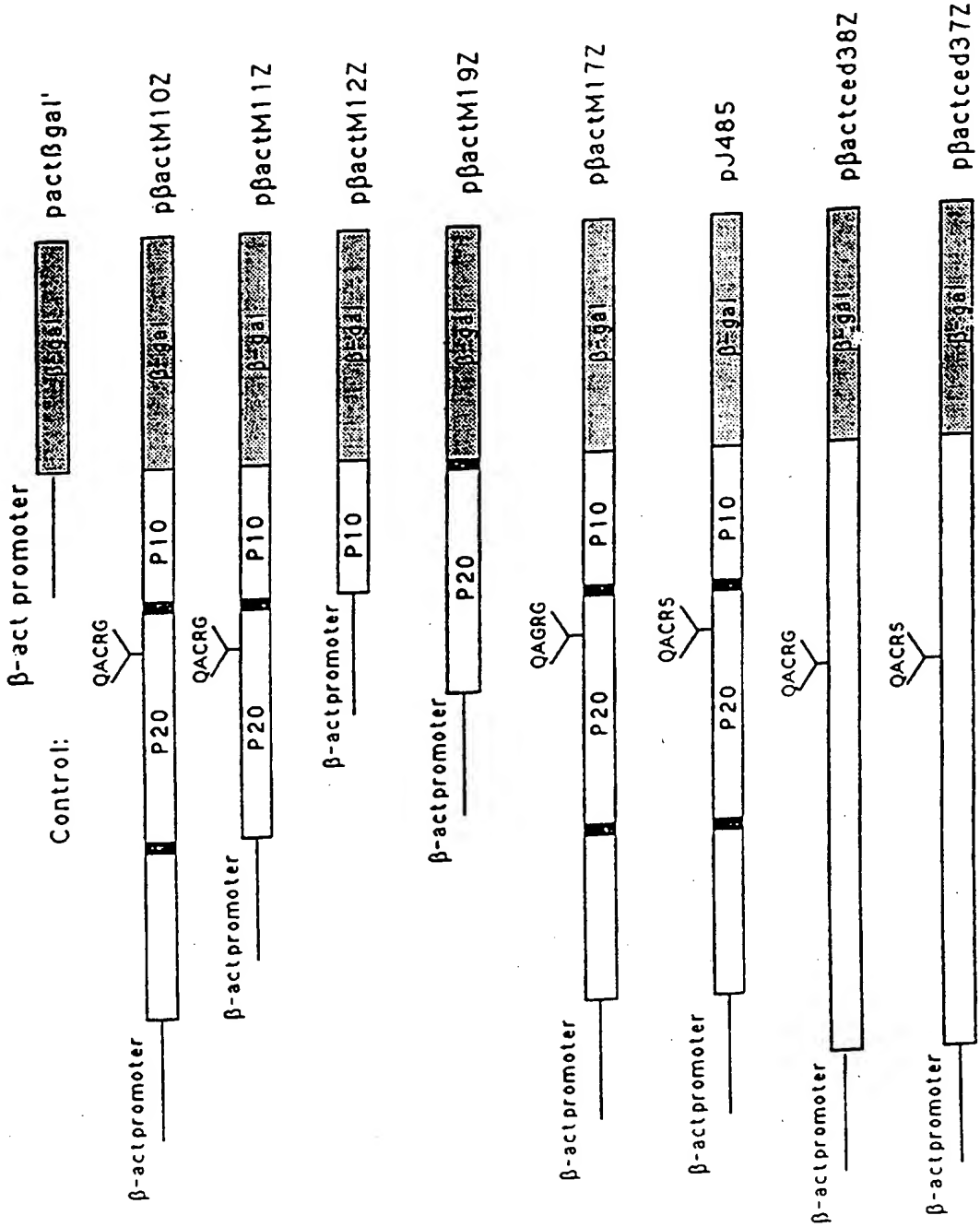
Control:
β-act promoter
pactβgal'
QACRG
P20
P10
β-actpromoter
pβactM10Z
pβactM11Z
pβactM12Z
pβactM19Z
QAGRG
pβactM17Z
QACRS
pJ485
pβactced38Z
pβactced37Z

FIGURE 4

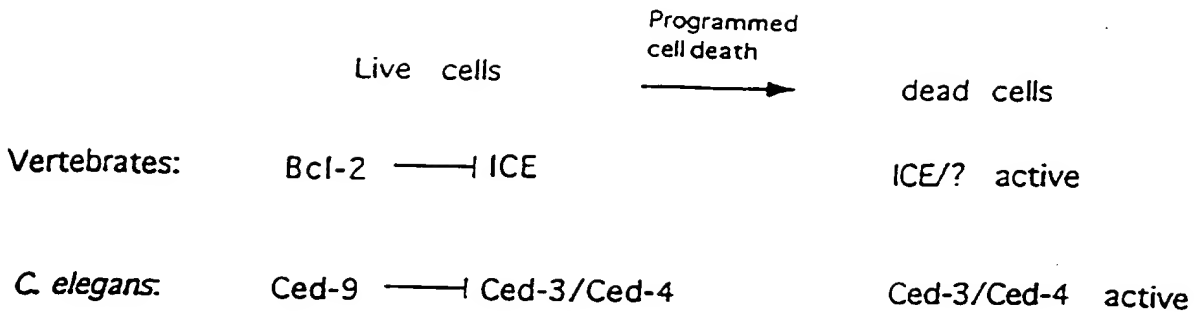
Programmed cell death
Live cells
dead cells
Vertebrates:
Bcl-2
ICE
ICE/? active
C. elegans:
Ced-9
Ced-3/Ced-4
Ced-3/Ced-4 active

FIGURE 5

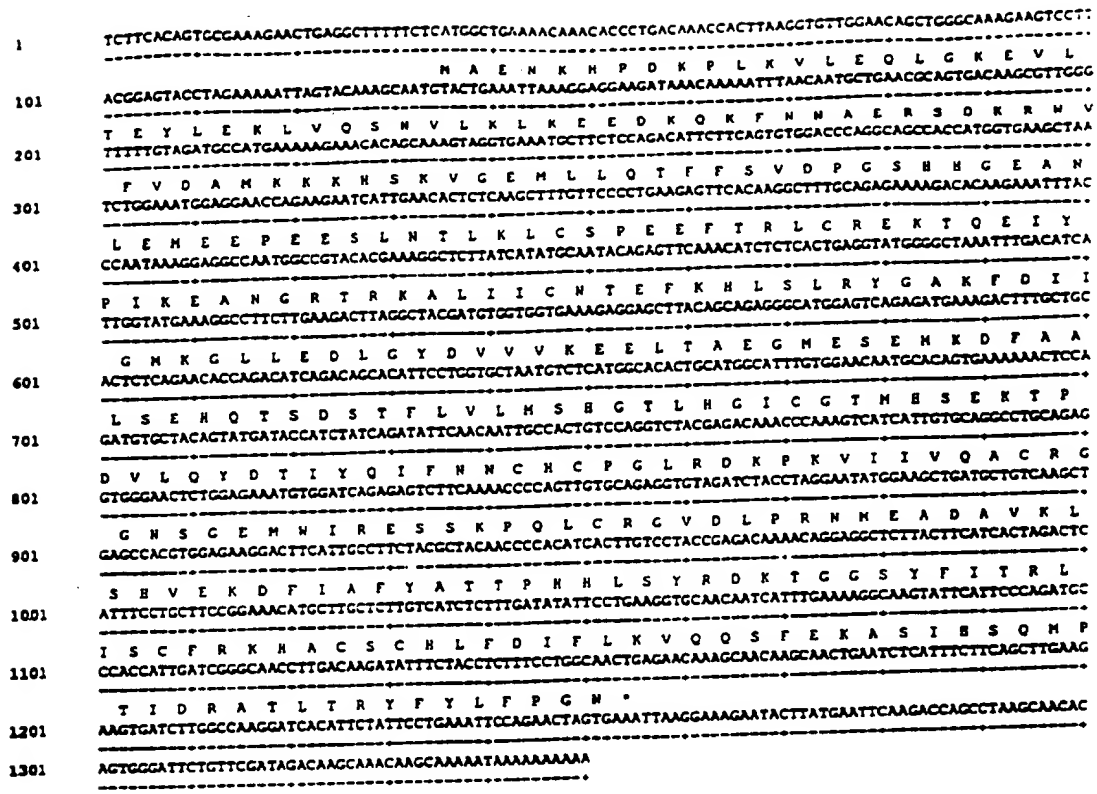

FIGURE 6

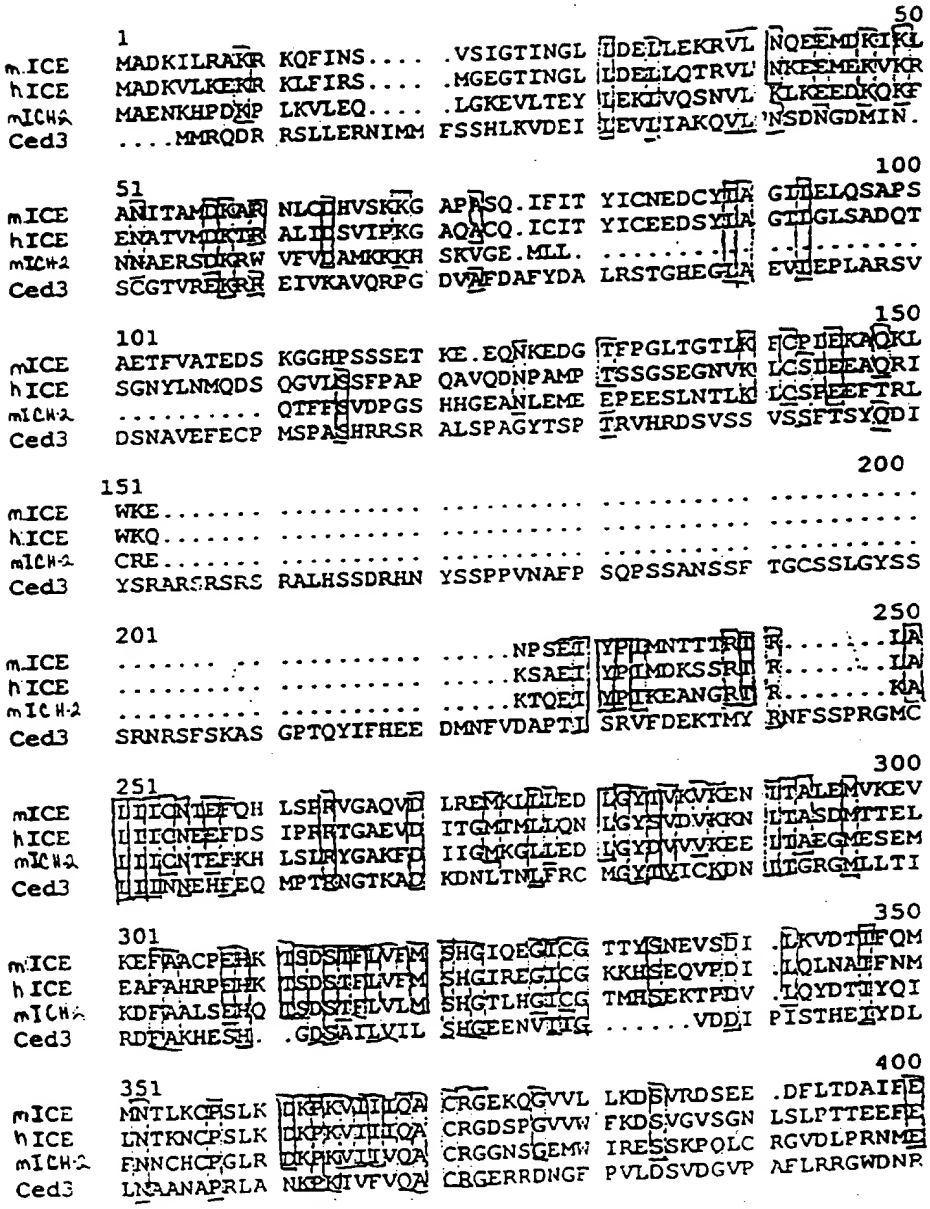
1 50
mICE MADKILRAKR KQFINS.... .VSIGTINGL IDELLEKRVL NQEEMDKIKL
hICE MADKVLKEKR KLFIRS.... .MGEGTINGL LDELLQTRVL NKEEMEKVKR
mICH2 MAENKHPDMP LKVLEQ.... .LGKEVLTEY LEKLVQSNVL KLKEEDKQKF
Ced3 ....MMRQDR RSLLERNIMM FSSHLKVDEI LEVLIAKQVL NSDNGDMIN.
51 100
mICE ANITAMDKAR NLQDHVSKKG APASQ.IFIT YICNEDCYLA GLLELQSAPS
hICE ENATVMDKTR ALLDSVIPKG AQACQ.ICIT YICEEDSYLA GLLGLSADQT
mICH2 NNAERSDKRW VFVLAMKKKH SKVGE.MLL. ....
Ced3 SCGTVREKRR EIVKAVQRPG DVAFDAFYDA LRSTGHEGLA EVLEPLARSV
101 150
mICE AETFVATEDS KGGHPSSSET KE.EQNKEDG TFPGLTGTLK EGPLEEKAQKL
hICE SGNYLNMQDS QGVLSSFPAP QAVQDNPAMP TSSGSEGNVK LGSLEEAQRI
mICH2 ..... QTFFSVDPGS HHGEANLEME EPEESLNTLK LGSPEEFTRL
Ced3 DSNAVEFECP MSPASHRRSR ALSPAGYTSP TRVHRDSVSS VSSFTSYQDI
151 200
mICE WKE..... ....
hICE WKQ..... ....
mICH2 CRE..... ....
Ced3 YSRARSRSRS RALHSSDRHN YSSPPVNAFP SQPSSANSSF TGCSSLGYSS
201 250
mICE ....NPSET YPLMNTTTRR R.....LA
hICE ....KSAET YPLMDKSSRR R.....LA
mICH2 ....KTQET MPLKEANGRR R.....KA
Ced3 SRNRSFSKAS GPTQYIFHEE DMNFVDAPTL SRVFDEKTMY RNFSSPRGMC
251 300
mICE LLICNTEFQH LSERVGAQVD LREMKLLLED LGYLVKVKEN LTALEMVKEV
hICE LLICNEEFDS IPERTGAEVD ITGMTMLLQN LGYSVDVKKN LTASDMTTEL
mICH2 LLICNTEFKH LSERYGAKFD IIGMKGLLED LGYDVVVKEE LTAEGMESEM
Ced3 LLINNEHEEQ MPTRNGTKAD KDNLTNLFRC MGYTVICKDN LTGRGMLLTI
301 350
mICE KEFAACPEHK LSDSTFLVFM SHGIQEGICG TTYSNEVSDI .LKVDTIFQM
hICE EAFAHRPEHK LSDSTFLVFM SHGIREGICG KKHSEQVPDI .LQLNAIFNM
mICH2 KDFAALSEHQ LSDSTFLVLM SHGTLHGICG TMHSEKTPDV .LQYDTIYQI
Ced3 RDFAKHESHL .GDSAILVIL SHGEENVIFG .....VDDI PISTHEIYDL
351 400
mICE MNTLKCPSLK GKPKVIIIQA CRGEKQGVVL LKDSVRDSEE .DFLTDAIFE
hICE LNTKNCPSLK GKPKVIIIQA CRGDSPGVVW FKDSVGVSGN LSLPTTEEFE
mICH2 FNNCHCPGLR GKPKVIIIVQA CRGGNSGEMW IRESSKPQLC RGVDLPRNME
Ced3 LNAANAPRLA NKPKIVFVQA CRGERRDNGF PVLDSVDGVP AFLRRGWDNR

FIGURE 7

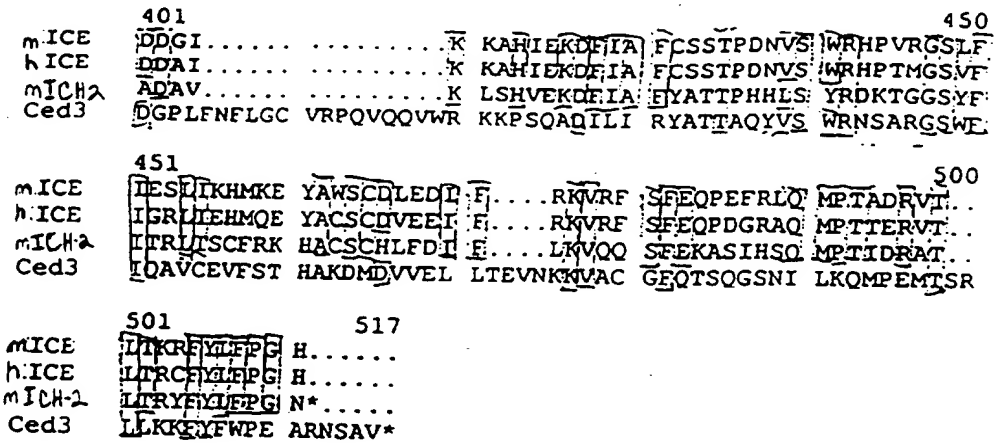
401
450
mICE DDGI..... .....K KAHIEKDFIA FCSSTPDNVS WRHPVRGSLF
hICE DDAI..... .....K KAHIEKDFIA FCSSTPDNVS WRHPTMGSVF
mICH2 ADAV..... .....K LSHVEKDFIA FYATTPHHLS YRDKTGGSYF
Ced3 DGPLFNFLGC VRPQVQQVWR KKPSQADILI RYATTAQYVS WRNSARGSWF
451
500
mICE IESLIKHMKE YAWSCDLEDI F....RKVRF SFEQPEFRLQ MPTADRVT..
hICE IGRLIEHMQE YACSCDVEEI F....RKVRF SFEQPDGRAQ MPTTERVT..
mICH2 ITRLTSCFRK HACSCHLFDI F....LKVQQ SFEKASIHSQ MPTIDRAT..
Ced3 IQAVCEVFST HAKDMDVVEL LTEVNKKVAC GEQTSQGSNI LKQMPEMTSR
501
517
mICE LTKRFYLFPG H.....
hICE LTRCFYLFPG H.....
mICH2 LTRYFYLFPG N*.....
Ced3 LLKKFYFWPE ARNSAV*

FIGURE 7A

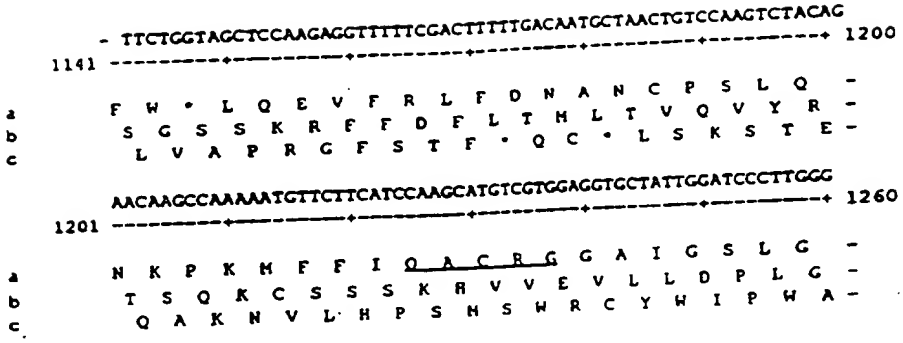
- TTCTGGTAGCTCCAAGAGGTTTTTCGACTTTTTGACAATGCTAACTGTCCAAGTCTACAG
1200
1141
F W * L Q E V F R L F D N A N C P S L Q -
S G S S K R F F D F L T H L T V Q V Y R -
L V A P R G F S T F * Q C * L S K S T E -
AACAAGCCAAAAATGTTCTTCATCCAAGCATGTCGTGGAGGTGCTATTGGATCCCTTGGG
1260
1201
N K P K M F F I Q A C R G G A I G S L G -
T S Q K C S S S K R V V E V L L D P L G -
Q A K N V L H P S M S W R C Y W I P W A -

FIGURE 8

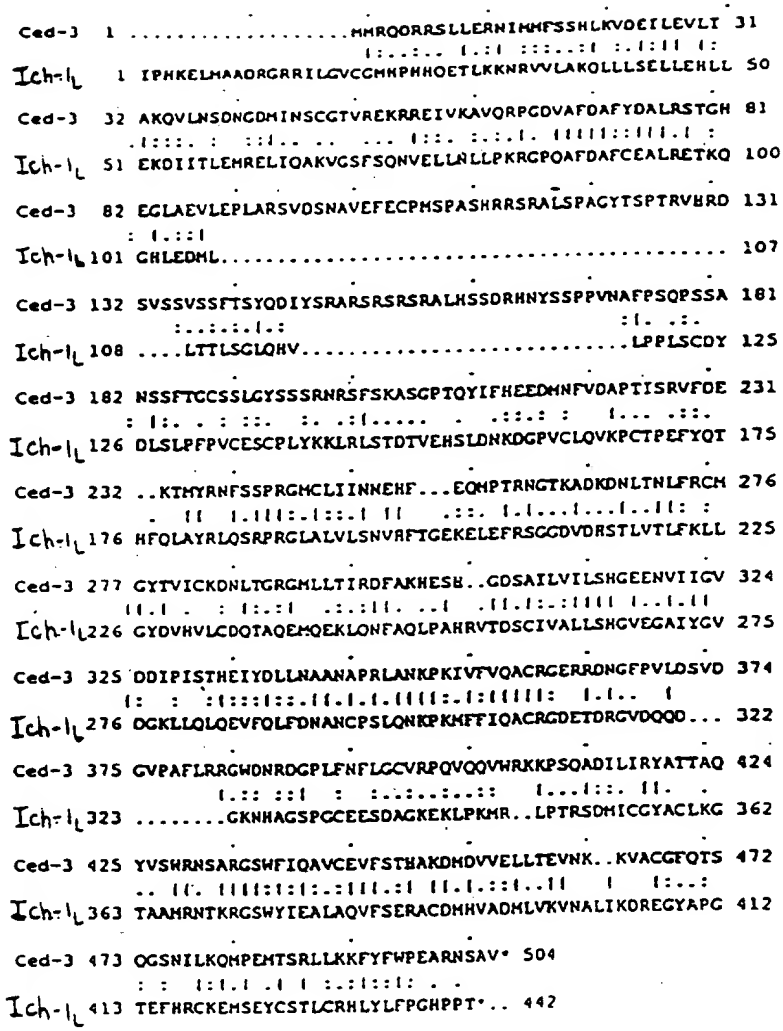
Ced-3 1 .....MMRQORRSLLERNIMMFSSHLKVQEILEVLI 31
Ich-1L 1 IPMKELMAADRGRRILGVCGMMPMMQETLKKNRVVLAKQLLLSELLEMLL 50
Ced-3 32 AKQVLNSDNGDMINSCGTVREKRREIVKAVQRPGDVAFDAFYDALRSTGH 81
Ich-1L 51 EKQIITLEMRELIQAKVGSFSQNVELLNLLPKRGPQAFDAFCEALRETKQ 100
Ced-3 82 EGLAEVLEPLARSVQSNAVEFECPMSPASMRRSRALSPAGYTSPTRVMRD 131
Ich-1L 101 GMLEQML..... 107
Ced-3 132 SVSSVSSFTSYQDIYSRARSRSRSRALMSSDRMNYSSPPVNAFPSQPSSA 181
Ich-1L 108 ....LTTLSGLQMV.....LPPLSCDY 125
Ced-3 182 NSSFTGCSSLGYSSSRNRSFSKASGPTQYIFMEEDMNFVDAPTISRVFDE 231
Ich-1L 126 DLSLPFPVCESCPLYKKLRLSTDTVEMSLDNKDGPVCLQVKPCTPEFYQT 175
Ced-3 232 ..KTMYRNFSSPRGMCLIINNEMF...EQMPTRNGTKADKDNLTNLFRCM 276
Ich-1L 176 MFQLAYRLQSRPRGLALVLSNVMFTGEKELEFRSGGDVDRSTLVTLFKLL 225
Ced-3 277 GYTVICKDNLTGRGMLLTIRDFAKMESM..GDSAILVILSHGEENVIIGV 324
Ich-1L 226 GYDVMVLCDQTAQEMQEKLQNFAQLPAMRVTDSCIVALLSHGVEGAIYGV 275
Ced-3 325 DDIPISTMEIYDLLNAANAPRLANKPKIVFVQACRGERRDNGFPVLDSVD 374
Ich-1L 276 DGKLLQLQEVFQLFDNANCPSLQNKPKMFFIQACRGDETDRGVDQQD... 322
Ced-3 375 GVPAFLRRGWDNRDGPLFNFLGCVRPQVQQVWRKKPSQADILIRYATTAQ 424
Ich-1L 323 .....GKNMAGSPGCEESDAGKEKLPKMR..LPTRSDMICGYACLKG 362
Ced-3 425 YVSMRNSARGSWFIQAVCEVFSTMAKDMDVVELLTEVNK..KVACGFQTS 472
Ich-1L 363 TAAMRNTKRGSWYIEALAQVFSERACDMMVADMLVKVNALIKDREGYAPG 412
Ced-3 473 QGSNILKQMPEMTSRLLKKFYFWPEARNSAV* 504
Ich-1L 413 TEFMRCKEMSEYCSTLCRMLYLFPGMPPT*.. 442

FIGURE 9

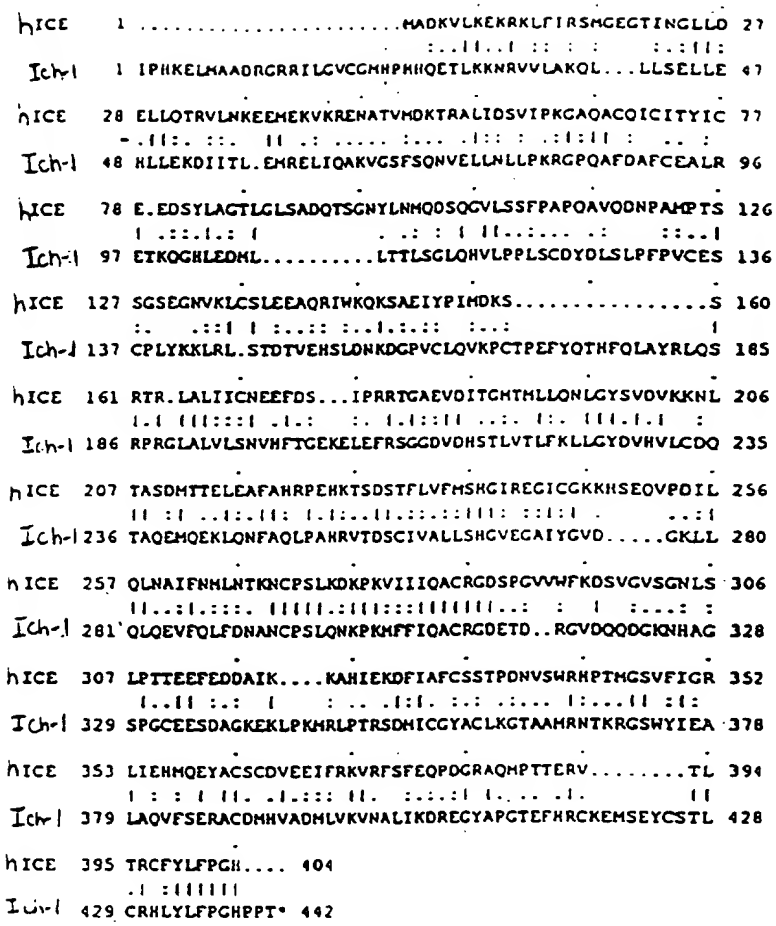
hICE 1 .....MADKVLKEKRKLFIRSMGEGTINGLLD 27
Ich-1 1 IPHKELMAADRGRRILGVCGMHPHHQETLKKNRVVLAKQL...LLSELLE 47
hICE 28 ELLQTRVLNKEEMEKVKRENATVMDKTRALIDSVIPKGAQACQICITYIC 77
Ich-1 48 KLLEKDIITL.EMRELIQAKVGSFSQNVELLNLLPKRGPQAFDAFCEALR 96
hICE 78 E.EDSYLAGTLGLSADQTSGNYLNMQDSQGVLSSFPAPQAVQDNPAMPTS 126
Ich-1 97 ETKQGHLEDML.....LTTLSGLQHVLPPLSCDYDLSLPFPVCES 136
hICE 127 SGSEGNVKLCSLEEAQRIWKQKSAEIYPIMDKS.....S 160
Ich-1 137 CPLYKKLRL.STDTVEHSLQNKDGPVCLQVKPCTPEFYQTHFQLAYRLQS 185
hICE 161 RTR.LALIICNEEFDS...IPRRTGAEVDITGMTMLLQNLGYSVDVKKNL 206
Ich-1 186 RPRGLALVLSNVHFTGEKELEFRSGGDVDHSTLVTLFKLLGYDVHVLCDQ 235
hICE 207 TASDMTTELEAFAHRPEHKTSDSTFLVFMSHGIREGICGKKHSEQVPDIL 256
Ich-1 236 TAQEMQEKLQNFAQLPAHRVTDSCIVALLSHGVEGAIYGVD.....GKLL 280
hICE 257 QLNAIFNMLNTKNCPSLKDKPKVIIIQACRGDSPGVVWFKDSVGVSGNLS 306
Ich-1 281 QLQEVFQLFDNANCPSLQNKPKMFFIQACRGDETD..RGVDQQQDGKNHAG 328
hICE 307 LPTTEEFEDDAIK....KAHIEKDFIAFCSSTPDNVSWRHPTMGSVFIGR 352
Ich-1 329 SPGCEESDAGKEKLPKMRLPTRSDMICGYACLKGTAAMRNTKRGSWYIEA 378
hICE 353 LIEHMQEYACSCDVEEIFRKVRFSFEQPDGRAQMPTTERV.....TL 394
Ich-1 379 LAQVFSERACDMHVADMLVKVNALIKDRECYAPGTEFHRCKEMSEYCSTL 428
hICE 395 TRCFYLFPGH.... 404
Ich-1 429 CRHLYLFPGHPPT* 442

FIGURE 9A

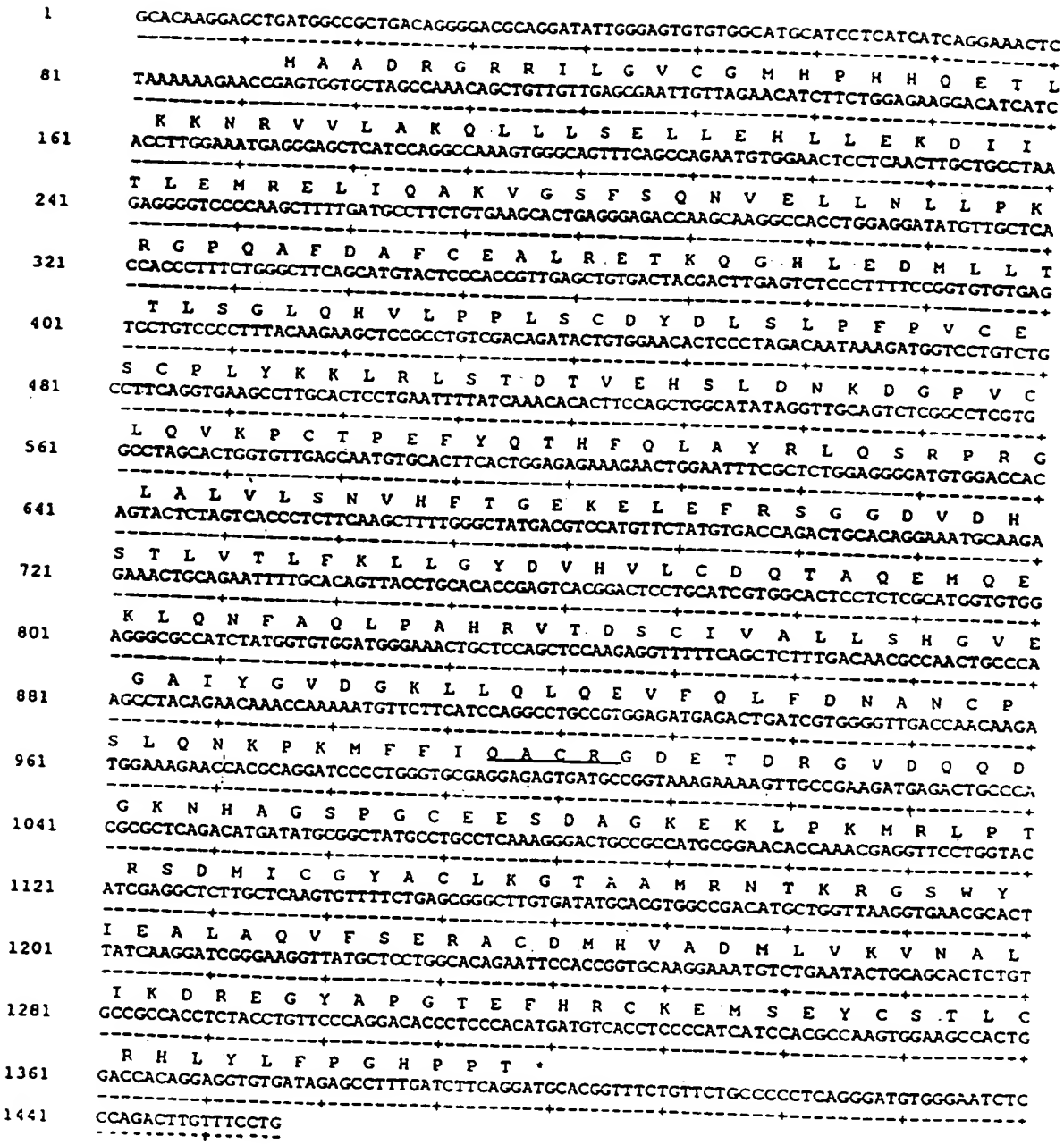
1
GCACAAGGAGCTGATGGCCGCTGACAGGGGACGCAGGATATTGGGAGTGTGTGGCATGCATCCTCATCATCAGGAAACTC
M A A D R G R R I L G V C G M H P H H Q E T L
81
TAAAAAAGAACCGAGTGGTGCTAGCCAAACAGCTGTTGTTGAGCGAATTGTTAGAACATCTTCTGGAGAAGGACATCATC
K K N R V V L A K Q L L L S E L L E H L L E K D I I
161
ACCTTGGAAATGAGGGAGCTCATCCAGGCCAAAGTGGGCAGTTTCAGCCAGAATGTGGAACTCCTCAACTTGCTGCCTAA
T L E M R E L I Q A K V G S F S Q N V E L L N L L P K
241
GAGGGGTCCCCAAGCTTTTGATGCCTTCTGTGAAGCACTGAGGGAGACCAAGCAAGGCCACCTGGAGGATATGTTGCTCA
R G P Q A F D A F C E A L R E T K Q G H L E D M L L T
321
CCACCCTTTCTGGGCTTCAGCATGTACTCCCACCGTTGAGCTGTGACTACGACTTGAGTCTCCCTTTTCCGGTGTGTGAG
T L S G L Q H V L P P L S C D Y D L S L P F P V C E
401
TCCTGTCCCCTTTACAAGAAGCTCCGCCTGTCGACAGATACTGTGGAACACTCCCTAGACAATAAAGATGGTCCTGTCTG
S C P L Y K K L R L S T D T V E H S L D N K D G P V C
481
CCTTCAGGTGAAGCCTTGCACTCCTGAATTTTATCAAACACACTTCCAGCTGGCATATAGGTTGCAGTCTCGGCCTCGTG
L Q V K P C T P E F Y Q T H F Q L A Y R L Q S R P R G
561
GCCTAGCACTGGTGTTGAGCAATGTGCACTTCACTGGAGAGAAAGAACTGGAATTTCGCTCTGGAGGGGATGTGGACCAC
L A L V L S N V H F T G E K E L E F R S G G D V D H
641
AGTACTCTAGTCACCCTCTTCAAGCTTTTGGGCTATGACGTCCATGTTCTATGTGACCAGACTGCACAGGAAATGCAAGA
S T L V T L F K L L G Y D V H V L C D Q T A Q E M Q E
721
GAAACTGCAGAATTTTGCACAGTTACCTGCACACCGAGTCACGGACTCCTGCATCGTGGCACTCCTCTCGCATGGTGTGG
K L Q N F A Q L P A H R V T D S C I V A L L S H G V E
801
AGGGCGCCATCTATGGTGTGGATGGGAAACTGCTCCAGCTCCAAGAGGTTTTTCAGCTCTTTGACAACGCCAACTGCCCA
G A I Y G V D G K L L Q L Q E V F Q L F D N A N C P
881
AGCCTACAGAACAAACCAAAAATGTTCTTCATCCAGGCCTGCCGTGGAGATGAGACTGATCGTGGGGTTGACCAACAAGA
S L Q N K P K M F F I Q A C R G D E T D R G V D Q Q D
961
TGGAAAGAACCACGCAGGATCCCCTGGGTGCGAGGAGAGTGATGCCGGTAAAGAAAAGTTGCCGAAGATGAGACTGCCCA
G K N H A G S P G C E E S D A G K E K L P K M R L P T
1041
CGCGCTCAGACATGATATGCGGCTATGCCTGCCTCAAAGGGACTGCCGCCATGCGGAACACCAAACGAGGTTCCTGGTAC
R S D M I C G Y A C L K G T A A M R N T K R G S W Y
1121
ATCGAGGCTCTTGCTCAAGTGTTTTCTGAGCGGGCTTGTGATATGCACGTGGCCGACATGCTGGTTAAGGTGAACGCACT
I E A L A Q V F S E R A C D M H V A D M L V K V N A L
1201
TATCAAGGATCGGGAAGGTTATGCTCCTGGCACAGAATTCCACCGGTGCAAGGAAATGTCTGAATACTGCAGCACTCTGT
I K D R E G Y A P G T E F H R C K E M S E Y C S T L C
1281
GCCGCCACCTCTACCTGTTCCCAGGACACCCTCCCACATGATGTCACCTCCCCATCATCCACGCCAAGTGGAAGCCACTG
R H L Y L F P G H P P T *
1361
GACCACAGGAGGTGTGATAGAGCCTTTGATCTTCAGGATGCACGGTTTCTGTTCTGCCCCCTCAGGGATGTGGGAATCTC
1441
CCAGACTTGTTTCCTG

Figure 10A

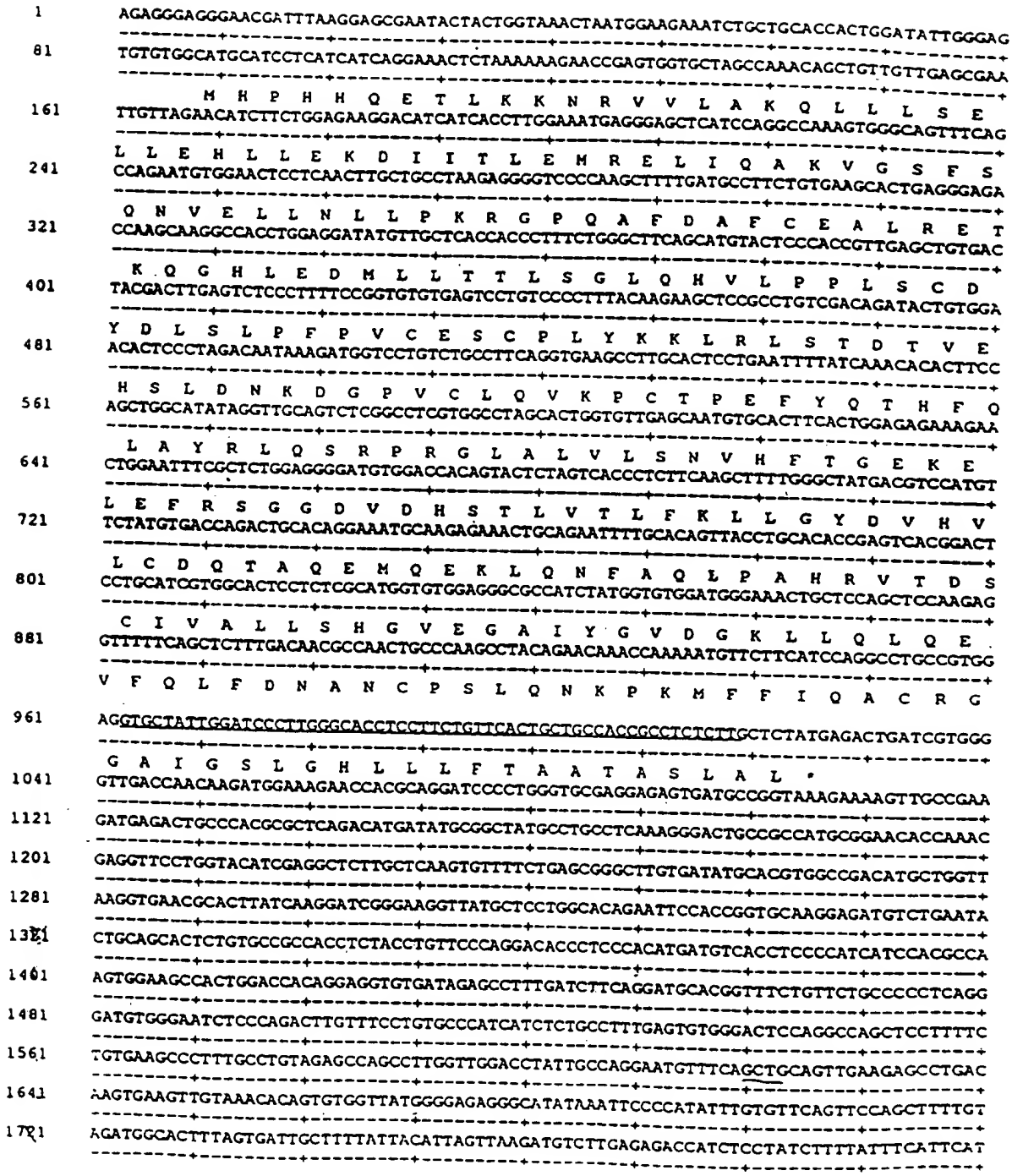

Figure 10 B

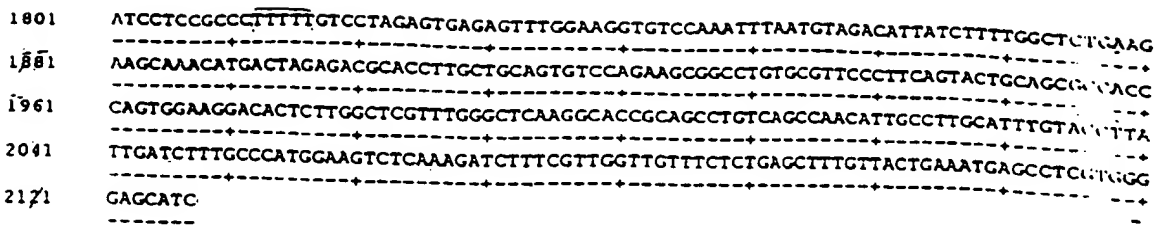

Figure 10B (cont'd)

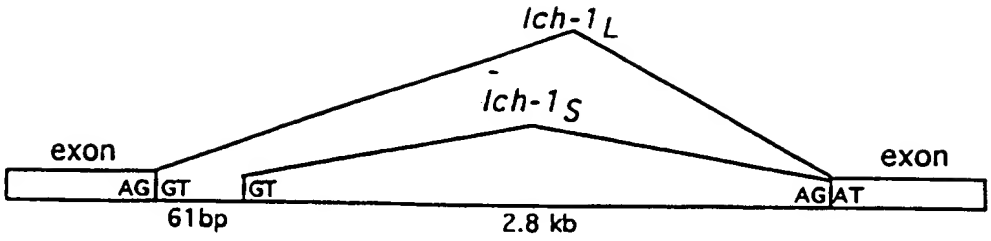

Figure 11A

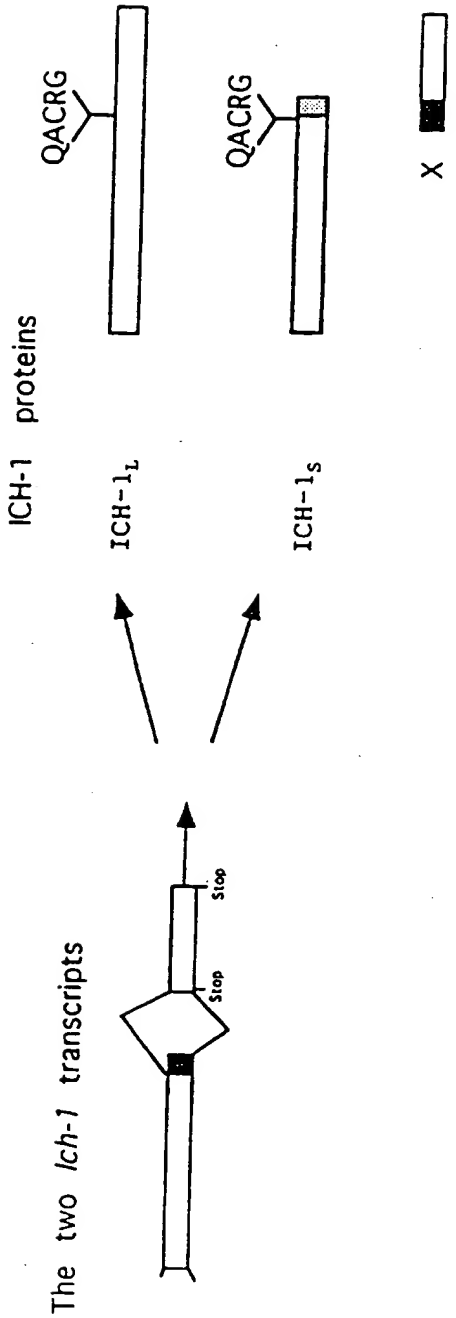
ICH-1 proteins
QACRG
QACRG
X
ICH-1L
ICH-1S
The two Ich-1 transcripts
Stop
Stop

Figure 11B

```
ICH-1S  ..............MHPHHQETLKKNRVVLAKQLLLSELLEHLLEKDIITLEMRELIQ.AKVGSFSQNVELLNLL 60
ICH-1L  MAADRGRRILGVCGMHPHHQETLKKNRVVLAKQLLLSELLEHLLEKDIITLEMRELIQ.AKVGSFSQNVELLNLL 74
hICE    MAD.........KVLKEKRKLFIRS.....MGEGTINGLLDELLQTRVLNKEEMEKVKRENATVMDKTRALIDSV 61
mICE    MAD.........KILRAKRKQFINS.....VSIGTINGLLDELLEKRVLNQEEMDKIKLANITAMDKARNLCDHV 61
Ced-3   ..............MMRQDRRSLLERNIMMFSSHLKVDEILEVLIAKQVLNSDNGDMIN.SCGTVREKRREIVKAV 61

ICH-1S  PKRGPQAFDAFCEALRETKQGHLEDML.....................................................L 88
ICH-1L  PKRGPQAFDAFCEALRETKQGHLEDML.....................................................L 102
hICE    IPKGAQACQ.ICITYICEEDSYLAGTLGLSADQTSGNYLNMQ.....................DSQGVL 108
mICE    SKKGAPASQ.IFITYICNEDCYLAGILELQSAPSAETFVATE.....................DSKGGH 108
Ced-3   QRPGDVAFDAFYDALRSTGHEGLAEVLEPLARSVDSNAVEFECPMSPASHRRSRALSPAGYTSPTRVHRDSVSSV 136

ICH-1S  TTLSGLQHVL.....................................PPLSCDYDLSLPFPVCESCPLYKKLRLSTDTVEHS 133
ICH-1L  TTLSGLQHVL.....................................PPLSCDYDLSLPFPVCESCPLYKKLRLSTDTVEHS 147
hICE    SSFPAPQAVQ.....................................DNPAMPTSSGSEGNVKLC.......SLEEA....QRI 144
mICE    PSSSETKE.E.....................................QNKEDGTFPGLTGTLKFC.......PLEKA....QKL 143
Ced-3   SSFTSYQDIYSRARSRSRSRALHSSDRHNYSSPPVNAFPSQPSSANSSFTGCSSLGYSSSRNRSFSKASGPTQYI 211

ICH-1S  LDNKDGPVCLQVKPCTPEFYQTHFQLAYRLQSRPRGLALVLSNVEFTGEKELEFRSGGDVDHSTLVTLFKLLGYD 208
ICH-1L  LDNKDGPVCLQVKPCTPEFYQTHFQLAYRLQSRPRGLALVLSNVEFTGEKELEFRSGGDVDHSTLVTLFKLLGYD 222
hICE    WKQ......KSAEIYPIMDKSSR....TR.......LALIICNEEF...DSIPRRTGAEVDITGMTMLLQNLGYS 199
mICE    WKE......NPSEIYPIMNTTTR....TR.......LALIICNTEF...QHLSPRVGAQVDLREMKLLLEDLGYT 196
Ced-3   FHEEDMNFVDAPTISRVFDEKTM....YRNFSSPRGMCLIINNEHF...EQMPTRNGTKADKDNLTNLFRCMGYT 279

Nedd-2                                          MLTVQV.YRTSQK.....CSSSKHVVEVLLDPLGTSFC.SL 36
ICH-1S  VHVLCDQTAQEMQEKLQNFAQLPAERVTDSCIVALLSHGVEGAIYG......VDGKLLQLQEVFQLFDNANCPSL 277
ICH-1L  VHVLCDQTAQEMQEKLQNFAQLPAERVTDSCIVALLSHGVEGAIYG......VDGKLLQLQEVFQLFDNANCPSL 291
hICE    VDVKKNLTASDMTTELEAFARRPEHKTSDSTFLVFMSEGIREGICGKKHSEQVPDI.LQLNAIFNMLNTKNCPSL 273
mICE    VKVKENLTALEMVKEVKEFAACPEHKTSDSTFLVFMSEGIQEGICGTTYSNEVSDI.LKVDTIFQMMNTLKCPSL 272
Ced-3   VICKDNLTGRGMLLTIRDFAKHESH..GDSAILVILSHGEENVIIG......VDDIPISTHEIYDLLNAANAPRL 346

                    S                                                          T
Nedd-2  LPPPLLLY.......ETDRGVDQQDGKNHTQSP.........GCEESDAG.....KEELMKMRLPTRSDMICGYAC 89
ICH-1S  QNKPKMFFIQACRGGAIGSLGHLLLFTAATASL.........AL*                          312
ICH-1L  QNKPKMFFIQACRGDETDRGVDQQDGKNHAGSP.........GCEESDAG.....KEKLPKMRLPTRSDMICGYAC 353
hICE    KDKPKVIIIQACRGDSPGVVW.FKDSV.............GVSGNLSLPTTEEFEDDAI.KKAHIEKDFIAFCSS 333
mICE    KDKPKVIIIQACRGEKQGVVL.LKDSV.............RDSEE.DFLTDAIFEDDGI.KKAHIEKDFIAFCSS 331
Ced-3   ANKPKIVFVQACRGERRDNGFPVLDSVDGVPAFLRRGWDNRDGPLFNFLGCVRPQVQQVWRKKPSQADILIRYAT 421

Nedd-2  LKGNAAMRNTKRGSWYIEALTQVFSERACDMEVADMLVKVNALIK.EREGYAPGTEFEHRCKEMSEYCSTLCQQLY 163
ICH-1L  LKGTAAMRNTKRGSWYIEALQVFSERACDMEVADMLVKVNALIK.DREGYAPGTEFEHRCKEMSEYCSTLCRHLY 427
hICE    TPDNVSWRHPTMGSVFIGRLIEHMQEYACSCDVEEIF....RKVRFSFEQPDGRAQMPTTERVT.....LTRCFY 399
mICE    TPDNVSWRHPVRGSLFIESLIKHMKEYAWSCDLEDIF....RKVRFSFEQPEFRLQMPTADRVT.....LTKRFY 397
Ced-3   TAQYVSWRNSARGSWFIQAVCEVFSTHAKDMDVVELLTEVNKKVACGFQTSQGSNILKQMPEMTSR...LLKKFY 493

Nedd-2  LFPGYPPT*  171
ICH-1L  LFPGHPPT*  435
hICE    LFPGH*     404
mICE    LFPGH*     402
Ced-3   FWPEARNSAV* 503
```

Figure 12A

(B) 27/33

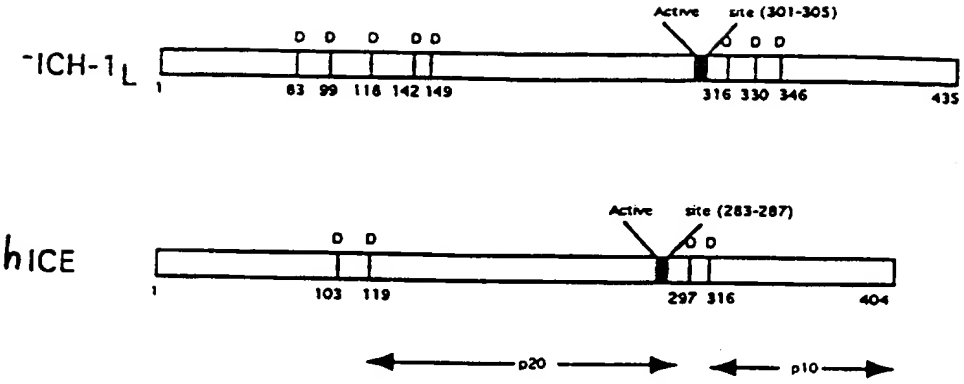

Figure 12B

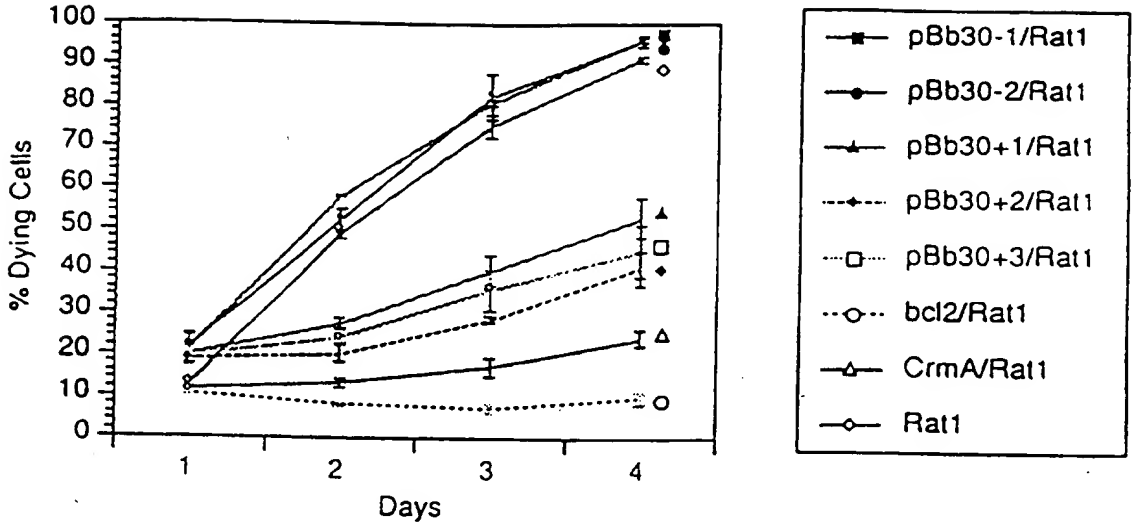
% Dying Cells
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
Days
pBb30-1/Rat1
pBb30-2/Rat1
pBb30+1/Rat1
pBb30+2/Rat1
pBb30+3/Rat1
bcl2/Rat1
CrmA/Rat1
Rat1

Figure 13

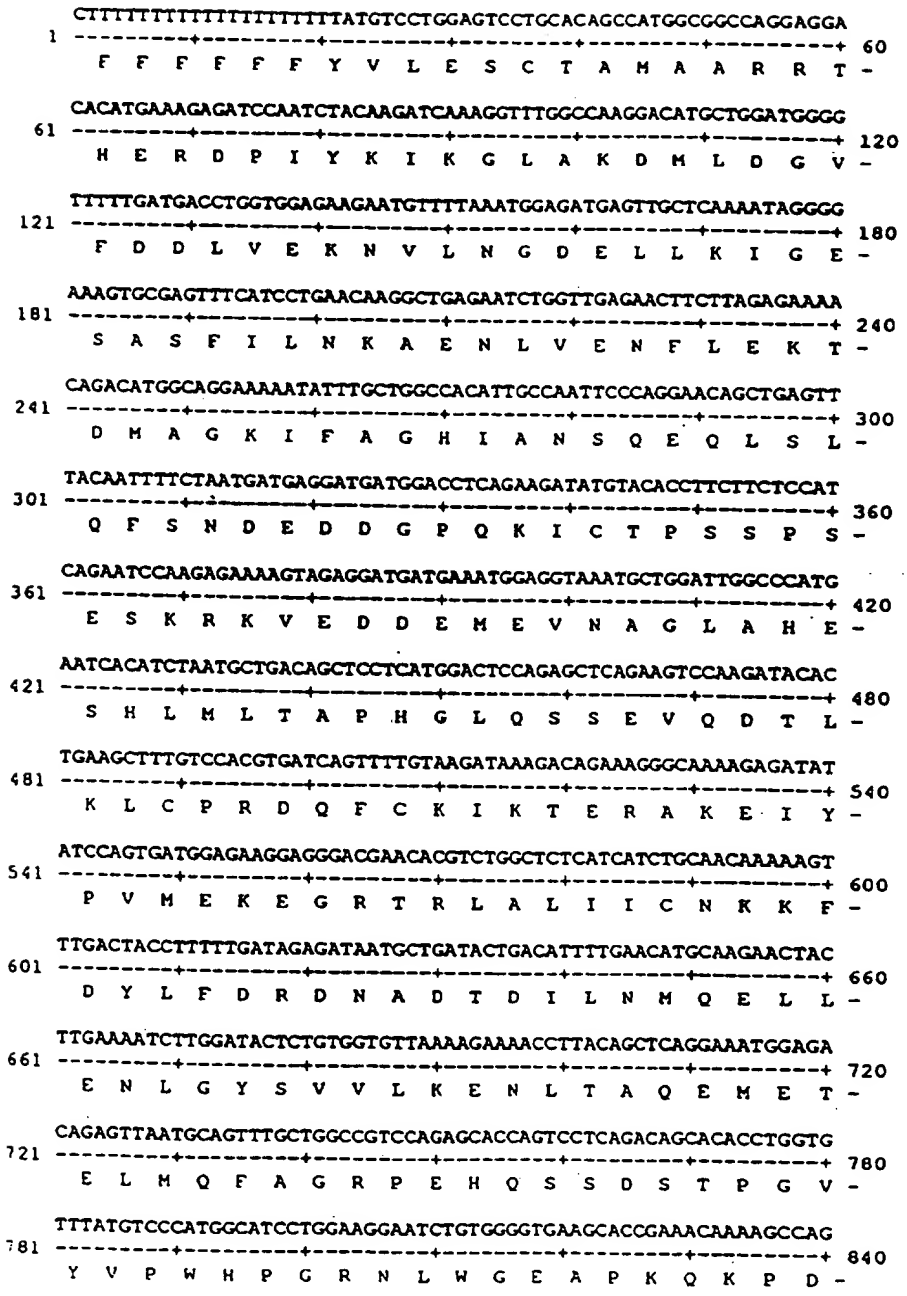
CTTTTTTTTTTTTTTTTTTTTATGTCCTGGAGTCCTGCACAGCCATGGCGGCCAGGAGGA
1
60
F F F F F F Y V L E S C T A M A A R R T -
CACATGAAAGAGATCCAATCTACAAGATCAAAGGTTTGGCCAAGGACATGCTGGATGGGG
61
120
H E R D P I Y K I K G L A K D M L D G V -
TTTTTGATGACCTGGTGGAGAAGAATGTTTTAAATGGAGATGAGTTGCTCAAAATAGGGG
121
180
F D D L V E K N V L N G D E L L K I G E -
AAAGTGCGAGTTTCATCCTGAACAAGGCTGAGAATCTGGTTGAGAACTTCTTAGAGAAAA
181
240
S A S F I L N K A E N L V E N F L E K T -
CAGACATGGCAGGAAAAATATTTGCTGGCCACATTGCCAATTCCCAGGAACAGCTGAGTT
241
300
D M A G K I F A G H I A N S Q E Q L S L -
TACAATTTTCTAATGATGAGGATGATGGACCTCAGAAGATATGTACACCTTCTTCTCCAT
301
360
Q F S N D E D D G P Q K I C T P S S P S -
CAGAATCCAAGAGAAAAGTAGAGGATGATGAAATGGAGGTAAATGCTGGATTGGCCCATG
361
420
E S K R K V E D D E M E V N A G L A H E -
AATCACATCTAATGCTGACAGCTCCTCATGGACTCCAGAGCTCAGAAGTCCAAGATACAC
421
480
S H L M L T A P H G L Q S S E V Q D T L -
TGAAGCTTTGTCCACGTGATCAGTTTTGTAAGATAAAGACAGAAAGGGCAAAAGAGATAT
481
540
K L C P R D Q F C K I K T E R A K E I Y -
ATCCAGTGATGGAGAAGGAGGGACGAACACGTCTGGCTCTCATCATCTGCAACAAAAAGT
541
600
P V M E K E G R T R L A L I I C N K K F -
TTGACTACCTTTTTGATAGAGATAATGCTGATACTGACATTTTGAACATGCAAGAACTAC
601
660
D Y L F D R D N A D T D I L N M Q E L L -
TTGAAAATCTTGGATACTCTGTGGTGTTAAAAGAAAACCTTACAGCTCAGGAAATGGAGA
661
720
E N L G Y S V V L K E N L T A Q E M E T -
CAGAGTTAATGCAGTTTGCTGGCCGTCCAGAGCACCAGTCCTCAGACAGCACACCTGGTG
721
780
E L M Q F A G R P E H Q S S D S T P G V -
TTTATGTCCCATGGCATCCTGGAAGGAATCTGTGGGGTGAAGCACCGAAACAAAAGCCAG
781
840
Y V P W H P G R N L W G E A P K Q K P D -

Figure 14

```
     ATGTTCTTCATGATGACACTATCTTCAAAATTTTCAACAACTCTAACTGTCGGAGTCTGA
841  ---------+---------+---------+---------+---------+---------+ 900
      V  L  H  D  D  T  I  F  K  I  F  N  N  S  N  C  R  S  L  R -

     GAAACAAACCCAAGATTCTCATCATGCAGGCCTGCAGAGGCAGATATAATGGAACTATTT
901  ---------+---------+---------+---------+---------+---------+ 960
      N  K  P  K  I  L  I  M  Q  A  C  R  G  R  Y  N  G  T  I  W -

     GGGTATCCACAAACAAAGGGATAGCCACTGCTGATACAGATGAGGAACGTGTGTTGAGCT
961  ---------+---------+---------+---------+---------+---------+ 1020
      V  S  T  N  K  G  I  A  T  A  D  T  D  E  E  R  V  L  S  C -

     GTAAATGGAATAATAGTATAACAAAGGCCCATGTGGAGACAGATTTCATTGCTTTCAAAT
1021 ---------+---------+---------+---------+---------+---------+ 1080
      K  W  N  N  S  I  T  K  A  H  V  E  T  D  F  I  A  F  K  S -

     CTTCTACCCCACATAATATTTCTTGGAAGGTAGGCAAGACTGGTTCCCTCTTCATTTCCA
1081 ---------+---------+---------+---------+---------+---------+ 1140
      S  T  P  H  N  I  S  W  K  V  G  K  T  G  S  L  F  I  S  K -

     AACTCATTGACTGCTTCAAAAAGTACTGTTGGTGTTATCATTTGGAGGAAATTTTTCGAA
1141 ---------+---------+---------+---------+---------+---------+ 1200
      L  I  D  C  F  K  K  Y  C  W  C  Y  H  L  E  E  I  F  R  K -

     AGGTTCAACACTCATTTGAGGTCCCAGGTGAACTGACCCAGATGCCCACTATTGAGAGAG
1201 ---------+---------+---------+---------+---------+---------+ 1260
      V  Q  H  S  F  E  V  P  G  E  L  T  Q  M  P  T  I  E  R  V -

     TATCCATGACACGCTATTTCTACCTTTTTCCCGGGAATTAGCACAGGCAACTCTCATGCA
1261 ---------+---------+---------+---------+---------+---------+ 1320
      S  M  T  R  Y  F  Y  L  F  P  G  N  *
     GTTCACAGTCAAGTATTGCTGTAGCTGAGAAGAAAAGAAAATTCCAAGATCCCAGGATTT
1321 ---------+---------+---------+---------+---------+---------+ 1380
     TTAAATGTGTAAAACTTTT
1381 ---------+--------- 1399
```

Figure 14 (cont'd)

```
                1                                                   50
ICH-3   ..........  .........M AARRTHERDP IYKIKGLAKD MLDGVFDDLV
hICE    ..........  .......... MADKILRAKR KQFINSVSIG TINGLLDELL
mICH-2  ..........  .......... MAENKHPDKP LKVLEQLGKE VLTEYLEKLV
ICH-1L  IPHKELMAAD  RGRRILGVCG MHPHHQETLK KNRVVLAKQL LLSELLEHLL
Ced3    ..........  .........M MRQDRRSLLE RNIMMFSSHL KVDEILEVLI

               51                                                  100
ICH-3   EKNVLNGDEL  LKIGESASFI LNKAENLVEN FLEKTDMAGK IFAGHI.ANS
hICE    EKRVLNQEEM  DKIKLANITA MDKARNLCDH VSKKGAPASQ IFITYI.CNE
mICH-2  QSNVLKLKEE  DKQKFNNAER SDKRWVFVDA MKKKHSKVGE MLL.......
ICH-1L  EKDIITLEMR  ELIQ.AKVGS FSQNVELLNL LPKRGPQAFD AFCEALRETK
Ced3    AKQVLNSDNG  DMIN.SCGTV REKRREIVKA VQRPGDVAFD AFYDALRSTG

               101                                                 150
ICH-3   QEQLSLQF..  ..........  ..........  ..........  ..........
hICE    DCYLAGIL..  ..........  ..........  ..........  ..........
mICH-2  ..........  ..........  ..........  ..........  ..........
ICH-1L  QGHLEDML..  ..........  ..........  ..........  ..........
Ced3    HEGLAEVLEP  LARSVDSNAV  EFECPMSPAS  HRRSRALSPA  GYTSPTRVHR

               151                                                 200
ICH-3   ....SNDEDD  GPQKICTPSS  PS........  ..........  ....ESKRKV
hICE    ....ELQSAP  SAETFVATED  SK........  ..........  ....GGHPSS
mICH-2  ..........  ..QTFFSVD.  .P........  ..........  ....GSHHGE
ICH-1L  .....LTTLS  GLQHV.....  ..........  ..........  ...LPPLSCD
Ced3    DSVSSVSSFT  SYQDIYSRAR  SRSRSRALHS  SDRHNYSSPP  VNAFPSQPSS

               201                                                 250
ICH-3   EDDEMEVNAG  LAHESHL...  MLTAPHGLQS  SEVQDTLKLC  PRDQFCKIKT
hICE    SETKEEQNKE  DGTFPGL...  T.........  ....GTLKFC  PLEKAQKLWK
mICH-2  ANLEMEEPEE  S.........  L.........  ....NTLKLC  SPEEFTRLCR
ICH-1L  YDLSLPFPVC  ESCPLYKKLR  LSTDTVEHSL  DNKDGPVCLQ  VKPCTPEFYQ
Ced3    ANSSFTGCSS  LGYSSSRNRS  FSKASGPTQY  IFHEEDMNFV  DAPTISRVFD

               251                                                 300
ICH-3   ERAKEIYPVM  EKEGRTRLAL  IICNKKF...  DYLFDRDNAD  TDILNMQELL
hICE    ENPSEIYPIM  NTTTRTRLAL  IICNTEF...  QHLSPRVGAQ  VDLREMKLLL
mICH-2  EKTQEIYPIK  EANGRTRKAL  IICNTEF...  KHLSLRYGAK  FDIIGMKGLL
ICH-1L  THFQLAYRLQ  SR..PRGLAL  VLSNVHFTGE  KELEFRSGGD  VDHSTLVTLF
Ced3    E..KTMYRNF  SS..PRGMCL  IINNEHF...  EQMPTRNGTK  ADKDNLTNLF

               301                                                 350
ICH-3   ENLGYSVVLK  ENLTAQEMET  ELMQFAGRPE  HQSSDSTPGV  YVPWHPGRNL
hICE    EDLGYTVKVK  ENLTALEMVK  EVKEFAACPE  HKTSDSTFLV  FMSHGIQEGI
mICH-2  EDLGYDVVVK  EELTAEGMES  EMKDFAALSE  HQTSDSTFLV  LMSHGTLHGI
ICH-1L  KLLGYDVHVL  CDQTAQEMQE  KLQNFAQLPA  HRVTDSCIVA  LLSHGVEGAI
Ced3    RCMGYTVICK  DNLTGRGMLL  TIRDFAKHES  H..GDSAILV  ILSHGEENVI

               351                                                 400
ICH-3   WGEAPKQK.P  DVLHDDTIFK  IFNNSNCRSL  RNKPKILIMQ  ACRGRYNGTI
hICE    CGTTYSNEVS  DILKVDTIFQ  MMNTLKCPSL  KDKPKVIIIQ  ACRGEKQGVV
mICH-2  CGTMHSEKTP  DVLQYDTIYQ  IFNNCHCPGL  RDKPKVIIVQ  ACRGGNSGEM
ICH-1L  YGVD.....G  KLLQLQEVFQ  LFDNANCPSL  QNKPKMFFIQ  ACRGDETDRG
Ced3    IGVD.....D  IPISTHEIYD  LLNAANAPRL  ANKPKIVFVQ  ACRGERRDNG
```

Figure- 15

```
         401                                                        450
ICH-3    WVSTNKGIAT A....DTDEE RVLSCKWNNS ITKAHVET.. .......DFI
hICE     LLKDSVRD.. .....SEEDF LTDAIFEDDG IKKAHIEK.. .......DFI
mICH-2   WIRESSKPQL C....RGVDL PRN..MEADA VKLSHVEK.. .......DFI
ICH-1L   VDQQD..... ......GKNH AGSPGCEESD AGKEKLPKMR ..LPTRSDMI
Ced3     FPVLDSVDGV PAFLRRGWDN RDGPLFNFLG CVRPQVQQVW RKKPSQADIL

         451                                                        500
ICH-3    AFKSSTPHNI SWKVGKTGSL FISKLIDCFK KYCWCYHLEE IFRKVQHSFE
hICE     AFCSSTPDNV SWRHPVRGSL FIESLIKHMK EYAWSCDLED IFRKVRFSFE
mICH-2   AFYATTPHHL SYRDKTGGSY FITRLISCFR KHACSCHLFD IFLKVQQSFE
ICH-1L   CGYACLKGTA AMRNTKRGSW YIEALAQVFS ERACDMHVAD MLVKVNALIK
Ced3     IRYATTAQYV SWRNSARGSW FIQAVCEVFS THAKDMDVVE LLTEVNK..K

         501                                                 540
ICH-3    VPGELTQMPT IERV..... ..SMTRYFYL FPGN*.....
hICE     QPEFRLQMPT ADRV..... ..TLTKRFYL FPGH.....
mICH-2   KASIHSQMPT IDRA..... ..TLTRYFYL FPGN*.....
ICH-1L   DREGYAPGTE FHRCKEMSEY CSTLCRHLYL FPGHPPT...
Ced3     VACGFQTSQG SNILKQMPEM TSRLLKKFYF WPEARNSAV*
```

Figure 15 (cont'd)

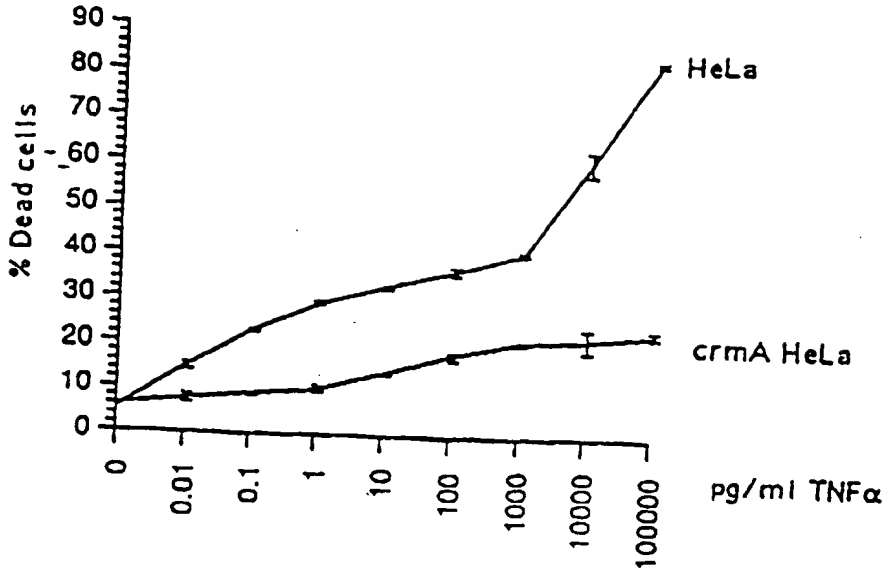
% Dead cells
90
80
70
60
50
40
30
20
10
0
HeLa
crmA HeLa
0
0.01
0.1
1
10
100
1000
10000
100000
pg/ml TNFα

Figure 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00177

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/12, 21, 44; 435/226, 320.1; 536/23.2, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21, 44; 435/226, 320.1; 536/23.2, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: ICE, interleukin 1beta converting enzyme, cell death, gene, crm-A

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAY et al., Viral Inhibition of Inflammation: Cowpox Virus Encodes an Inhibitor of the Interleukin-1beta Converting Enzyme. Cell. 15 May 1992, Vol.69, pages 597-604, see entire document.	1-3
A	THORNBERRY et al. A novel heterodimeric cysteine protease is required for interleukin-1beta processing in monocytes. Nature. 30 April 1992, Vol.356., pages 768-774, see entire document.	1-26
A	CERRETTI et al., Molecular Cloning of the Interleukin-1beta Converting Enzyme. Science. 03 April 1992, Vol. 256, pages 97-100, see entire document	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 APRIL 1996	06 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KEITH D. HENDRICKS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00177

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00, 38/46, 48/00; C07K 2/00, 4/00, 14/00, 16/00; C12N 15/57